Metabolic Control Mechanisms in Animal Cells

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METABOLIC CONTROL MECHANISMS IN ANIMAL CELLS

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It is a pleasure to acknowledge the contributions of the staff of the Journal of the National Cancer Institute to the preparation of this monograph.

PREFACE

The previous Tissue Culture Association symposia, "Analytic Cell Culture," National Cancer Institute Monograph 7, and "Symposium on Organ Culture," National Cancer Institute Monograph 11, have dealt with: 1) refinement of tissue culture methodology, 2) characterization of cultured cells and tissues, and 3) the application of *in vitro* culture techniques to the study of various physiological phenomena.

The present symposium is concerned with the problem of defining at the molecular level mechanisms of control of cell proliferation and metabolic function. Although many significant, relevant experiments have been performed with microorganisms, the symposium is primarily focused on experimentation in animal cells. The recent progress toward the elucidation of the structure and biosynthesis of deoxyribonucleic acid and ribonucleic acid, and their role in defining the structure and formation of proteins, together with the demonstration that small molecules (frequently substrates or substrate analogues) can selectively affect (repress or derepress) the synthesis of specific proteins, have stimulated fresh and optimistic approaches to the chemical analysis of complex regulatory processes such as cytodifferentiation and hormone action in multicellular organisms.

It is anticipated that simplified cellular systems will aid materially in the experimental analysis of these processes. However, no attempt has been made to restrict discussion to experiments employing tissue culture methods. Various aspects of these problems may be more appropriately investigated at other levels of cellular organization (or disorganization).

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PROTEIN SYNTHESIS IN DIFFERENTIATING CHICK SKIN[1,2]

EUGENE BELL,[3] Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

How cells arrive at their specialized functional state is, in broad terms, the subject of this session. Put in another way the question is: How do cells in a multicellular environment differentiate?

It is becoming increasingly clear that what a cell is, is expressed through that portion of its deoxyribonucleic acid (DNA) which is available for transcription. The key to differentiation lies in the explanation of how one pattern of available DNA sites is changed to another pattern. Whatever the causes, they would leave their mark at the level of the genetic machinery of the cell, changing not the DNA but those factors that control the pattern of its readout. Although some schemes have been proposed recently (*18*, *17*), we are still entirely ignorant of how the pattern of readout of DNA is controlled, changed, or perpetuated.

We might suspect that the proteins associated with DNA and the organization of the chromosome itself are the immediate regulating agents (*15*, *16*, *4*, *5*).

Our concern, also, is how and when does a changing embryonic environment play its tune on DNA; that is on the factors which control its expression. What, then, are the mechanisms of gene activation? Are the visibly catastrophic events of development, such as cleavage, gastrulation, or neurulation exclusively responsible for driving cells into new states of differentiation? Or is the process by which the pattern of expression of DNA is molded a continuous one? That is, do cells change in a few large jumps or in many small steps?

If we look at the epiphenomena, which are a consequence of applying successively different stencils of repression on the DNA, these questions seem more tangible. Perhaps only one step removed from the chromosomes, which may themselves be the central differentiating entities (*14*), and reflecting their progressively altered functional states in developing cells, is messenger ribonucleic acid (mRNA). By tracing changes in the species of mRNA it may be possible to tell certainly *not how*, but at least

[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27-30, 1963.

[2] Supported in part by grant GB from the National Science Foundation.

[3] The technical assistance of Miss Charlotte Merrill, Mrs. Pearl Goodwin, and Miss Elaine Pacetti is gratefully acknowledged.

when new proteins come on stage. It is still difficult to predict whether the appearance of only a few new kinds of messenger will be detectible against a background of many thousands, even with the possibilities of fractionating mRNA in a polysome gradient (*22*, *23*) and hybridizing it with DNA (*25*, *6*, *9*).

At the moment, there is only the alternative of viewing differentiation as a sequence of changes in the patterns of protein synthesis. But even these changes are difficult to recognize. With what precision, in looking at the time course of development, can we say that a protein is being synthesized for the first time?

There is some evidence that the proteins by which cells will be known may be synthesized long before those cells undergo morphological differentiation (*24*, *7*, *12*). Contrariwise, there is argument that specialized synthesis does not begin until cells look like, or nearly look like, their pictures in the *Textbook of Histology* (*21*, *11*).

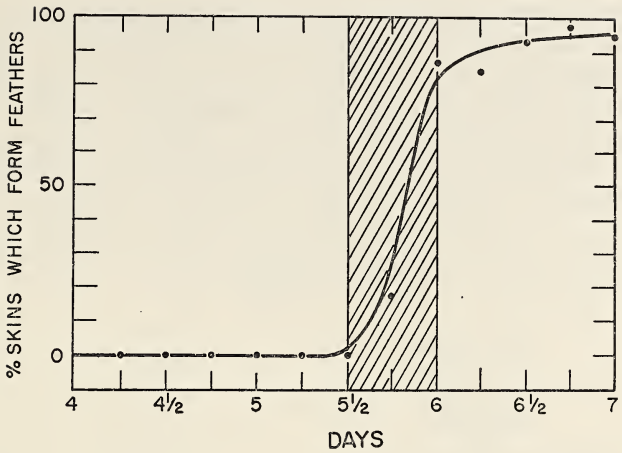
Disagreement probably stems from the absence of hard facts. "When does a protein appear for the first time?" The answer to the question depends on the sensitivity and specificity of the method used for its detection. The matter is further complicated by the difficulty of choosing the right protein or product to look at to evaluate how far a cell is from its final differentiated state.

Despite the foregoing handicaps, we have had the temerity to look at this question in studying the development of chick skin and feathers. I would like to talk briefly about two aspects of the work: 1) protein synthesis in relation to feather induction and 2) the problem of the recognition of a principal product by which the skin will be known, *i.e.*, keratin.

That an inductive event occurs in the development of the skin has already been demonstrated by Sengel (*19*). He showed that skins in organ culture would not differentiate if they were younger than 6¾ days of age. Older skins under the same conditions produced feathers and keratinized.

We have confirmed his results and refined the assay. Pieces of embryonic skin were removed from the backs of chick embryos of different ages. Each skin piece was mounted on a membrane filter raft and incubated in completely defined minimal medium containing no protein for 5 to 7 days. None of the skins 5½ days of age or younger formed feathers, only 17 percent of the skins that were 5¾ days of age formed feathers, while almost all skins 6 days of age or older formed feathers (text-fig. 1).

After 24 hours in culture, 7-day skins incorporated twice as much amino acid into protein as 5½-day or preinduced skins. Similarly, RNA synthesis after 24 hours of *in vitro* growth is twice as great in 7-day skins as in 5½-day skins, as judged by incorporation of uridine. After 5 days in culture, the differences are increased to threefold and fourfold, respectively, for protein and RNA synthesis. Hence, in the *in vitro* system, induced skin is metabolically far more active than uninduced skin. These results suggest that some time between 5½ and 6 days *in ovo* the skin is induced to make feathers. We have shown previously that the inducer which triggers



TEXT-FIGURE 1.—Differentiation of embryonic chick skin on defined medium as a function of age at the time of explantation. Only skins of 6 days and older could form feathers with high frequency. Each point represents 20 or more assays.

the differentiation of skin sometime between 5½ and 6 days *in vivo* can be mimicked *in vitro* by serum albumin very effectively and by some other proteins to a lesser extent (*1*).

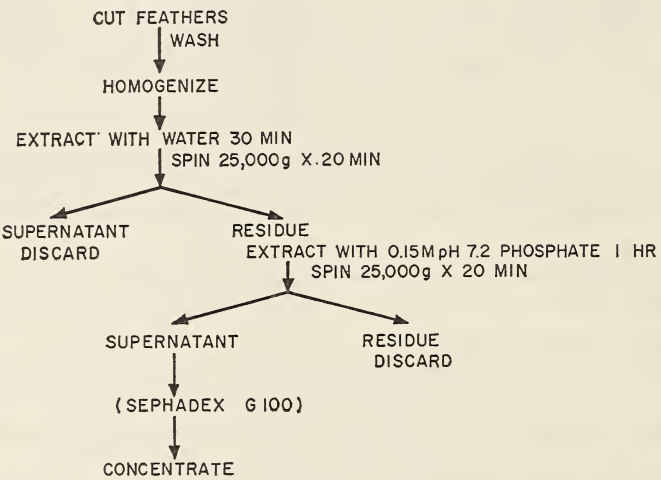
There is no doubt, operationally, that an inductive event occurs in the development of skin. Are there changes in protein synthesis which may be associated with it? It might be expected that after induction certain proteins will be synthesized at the expense of others, or that the synthesis of some other proteins might be terminated. Would keratin, for example, make its debut at the time of induction? One of the problems in answering the question was the identification of the protein.

Using X-ray diffraction criteria we have shown that it is not until sometime between the 13th and 14th day of incubation that the developing feather gives the completed pattern for adult keratin (*3*). We proposed, however, that certain reflections seen in the pattern of feathers aged 10 to 13 days may indeed be diagnostic for feather keratin. We decided then to look at the X-ray patterns of protein extracted from feathers younger than 14 days. Both X-ray and cytochemical data indicated that extensive cross linking of the protein occurred between the 13th and 14th day of development.

Hence, early 13-day feathers might be excellent material from which to extract keratin since it was yet only partially polymerized. With Dr. Ronald Malt and Mrs. Haruko Meyer, we developed an extraction procedure (text-fig. 2), which yielded soluble feather protein (*13*, *2*). When partially dried down and drawn into a fiber the 13-day protein gave an X-ray diffraction pattern which, though not identical, was similar to that of 14-day feathers or adult keratin.

Figures 1 and 2 are electron micrographs prepared according to the method of Hall (*10*) by Stewart (*20*) of the extracted protein. The

larger fibers appeared to consist of beads along a string. The estimated diameter of the bead is about 20 to 30 A (fig. 1). The smallest strands measured 12 A in width. By extraction with acetate buffer of lower ionic strength than that of the phosphate, a unit of consistent size (approximately 50 A) was observed (fig. 2). In some preparations of the protein extracted with phosphate buffer, cross-linked long chains or bundles of fibrils were seen. Thus, at 13 days there is already present a fibrous protein which is organized in a bead-like array. The reality of the bead is underscored by the fact that appropriate buffer dissociates the fiber into component beads.



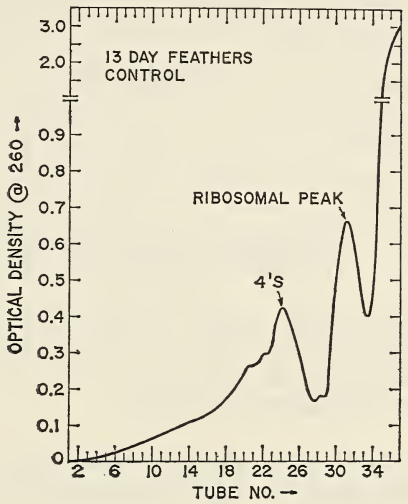
Text-figure 2.—Flow diagram of procedure for preparation of protein shown in figure 1.

Hence, we have been able to show that the X-ray pattern observed as early as 10 days, since it is essentially the same as that of 13-day feathers, is possibly already indicative of the presence of keratin in some organized form.

It is important to note that at this time feather cells are yet morphologically relatively undifferentiated and actively dividing. Nonetheless, they have already begun to synthesize proteins of which the final feather will consist. Other evidence from our laboratory would indicate that feather protein synthesis may begin earlier than 10 days. Dr. Ben-Or has found that antisera to 7-day ectoderm is nearly completely absorbed by 7-, 6-, and 5-day skin. Three-day skin, however, is incapable of completely absorbing it. Similarly, antisera to a guanidine extract of keratin from 13-day feathers are completely absorbed by not only 6- and 7-day skins but also by 5-day skins.[4] This data would suggest also that no change in the spectrum of immunologically detectible protein can be associated with the induction of feathers.

[4] Ben-Or, S., and Bell, E.: Ontogeny of skin antigens in the chick. In preparation.

It has been shown recently that the site of protein synthesis in the cytoplasm is the polyribosome or polysome (*22, 23*). An assembly of ribosomes becomes associated with mRNA and in this physical arrangement the mRNA codes functionally for protein. The polysome profile of 13-day feathers is shown in text-figure 3. This is an optical density profile at 260 mμ of the polysomes in a 15 to 30 percent sucrose gradient.[5]

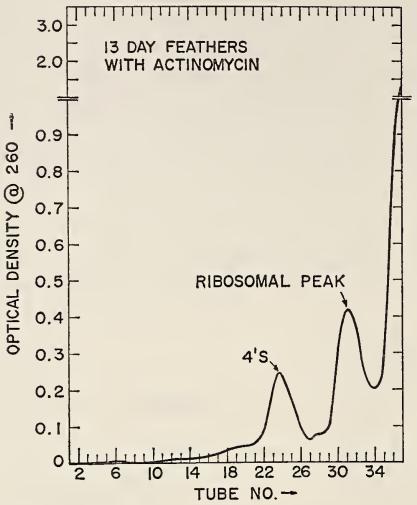


TEXT-FIGURE 3.—Polysome profile of 13-day feathers after 30 hours of *in vitro* incubation in the modified medium of Charity Waymouth. Polysomes are distributed in a 15 to 30 percent sucrose gradient. Although the 4's peak predominates, a substantial fraction of the total optical density is accounted for by material at the heavy end of the gradient.

Primarily, 2 peaks in addition to the 74S ribosomal peak are present: one which is due to aggregates of 3 ribosomes in association with messenger and nascent protein, and another which is due to aggregates of 4 ribosomes, again in association with mRNA and protein. This pattern remains essentially unchanged in skins of 5 to 16 days of incubation. As development proceeds, however, there appears to be a marked increase in the concentration of quatrasomes or of the 4's peak. It is with polysomes of this size that we suspect keratin messenger may be associated. When cut feathers are pulse-labeled with amino acids (algal hydrolysate) for 3 minutes *in vitro* and the distribution of radioactivity in the gradient plotted on the graph of the optical density profile, it is found that the radioactivity closely follows the optical density. Although we already believe that the protein associated with the quatrasomes is indeed keratin, further proof is required.

It is striking that when 13-day feathers are treated with actinomycin D, the 4's peak is practically unaffected (text-fig. 4). A decrease in the optical density, especially of the heavy end of the gradient, is, however,

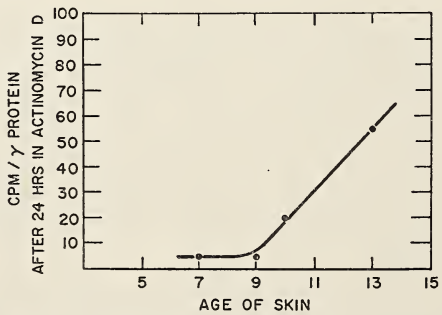
[5] Spun for 2 hours at 25,000 rpm; Spinco SW 25 head.



TEXT-FIGURE 4.—Polysome profile of 13-day feathers after 30 hours of incubation with actinomycin D (60 γ/ml) in modified medium of Charity Waymouth. The stable component is in the 4's peak. Most of the optical density due to polysomes elsewhere is reduced to the baseline value.

observed. Actinomycin D interferes with DNA mediated RNA synthesis (*8*) and hence would abruptly stop the synthesis of mRNA. Indeed, incorporation of C^{14}-uridine into actinomycin-treated feathers (10 γ/ml) after 2 hours is down to the 5 percent level when compared with that of controls. Both the optical density and radioactivity profiles for 13-day actinomycin D-treated feathers would indicate that messenger for feather protein appears to be relatively stable in 13-day feathers.

Text-figure 5 shows the sensitivity of skins of different ages to actinomycin D. Before 9 days, amino acid incorporation is reduced to the 5 to 7 percent level when skins are incubated for 24 hours in actinomycin D. After that it rises to about 60 percent in 13-days feathers and is probably higher in older material. Over the period of feather induction, between



TEXT-FIGURE 5.—Sensitivity of epidermis or feathers of different ages to 10 γ per ml actinomycin D. At 13 days (in feathers) nearly 60 percent protein synthesis is unaffected, while in 9-day epidermis virtually all protein synthesis is stopped.

5 and 7 days, no qualitative or quantitative change in the character of the polysome profile is observed. A marked change in the sensitivity to actinomycin D appears to occur at about 9 days, probably at a time when the synthesis of keratin is becoming more and more the exclusive activity of feather cells. The gradual decrease in the sensitivity to actinomycin D may be due to the possibility that increasing numbers of cells in an initially inhomogeneous population are making feather protein. Dr. Humphreys and Dr. Penman were collaborators in the polysome studies.

In summary, we have described an inductive event that occurs in skin and have been unable to show so far that synthesis of a new set of proteins or a change in the pattern of protein synthesis is associated with feather induction. We have seen that keratin, even in a highly organized form, appears in cells before they have arrived at their final morphological states and that in RNA for feather protein has a long half-life. Relatively early in feather development regulation of protein synthesis in some cells occurs at the level of RNA.

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Plate 1

Figure 1.—Electron micrograph of sprayed 13-day embryonic feather protein prepared by the shadow transfer technique. *Arrow* points to chain or "fibril" of smallest caliber. Chains join laterally to form fibers of larger caliber which show distinct nodularity. Polystyrene beads are 880 A diameter.

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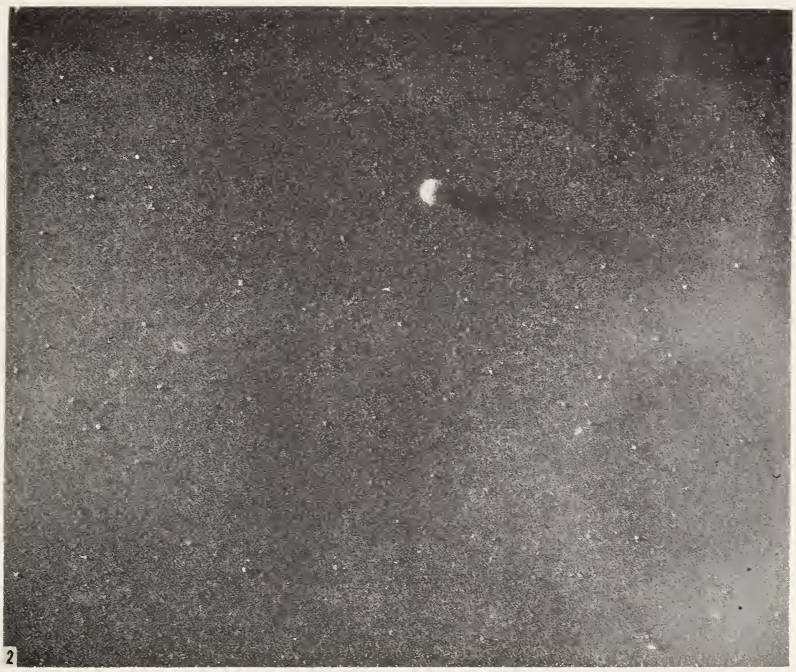
PLATE 2

FIGURE 2.—Electron micrograph of same protein as shown in figure 1 but extracted with buffer of lower molarity (acetate, 0.15 M, *p*H 7.2).


2

ISOLATION AND BIOLOGICAL EFFECTS OF AN EPIDERMAL GROWTH-STIMULATING PROTEIN [1, 2, 3]

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It has long been recognized that the growth and differentiation of certain specific cells are regulated by specific chemical substances produced in the organism. The extent to which the organism may rely on this mechanism has not, perhaps, been fully appreciated.

Two hitherto unsuspected growth-regulating factors have been studied in recent years in our laboratories: the "nerve growth factor" (NGF) and an "epidermal growth factor" (EGF). The discovery, isolation, and biological effects of this latter factor are reviewed here.

During our investigations on the nerve growth-promoting protein of the submaxillary gland of the mouse (*1*, *2*), we noted that the daily injection of partially purified extracts of the salivary gland into newborn mice resulted in some gross anatomical changes, in addition to the previously reported effects on the nerve cells: (*a*) precocious opening of the eyelids, as early as 7 days instead of the usual 12 to 14 days; (*b*) precocious eruption of the incisors, at 6 to 7 days instead of the normal 8 to 10 days; and (*c*) a marked stunting of the animals with an inhibition of hair growth.

With a biological assay based on the precocious opening of the eyelids in newborn mice, the purification and identification of the factor were attempted. The isolation of the factor was reported in 1962 (*3*), and it was found to be a heat-stable, nondialyzable, antigenic protein, whose most distinctive chemical characteristic was the absence of phenylalanine and lysine.

The isolation procedure involved standard methods of protein fractionation and included, in the final purification steps, chromatography on carboxymethylcellulose and diethylaminoethylcellulose and gel filtration with Sephadex G-75. From 22 g wet weight of submaxillary glands derived from 150 adult male mice, 5 to 7 mg of the pure protein was obtained. This represented a yield of approximately 20 percent, with a purification of about 150-fold, based on protein content.

[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27-30, 1963.

[2] This investigation was supported in part by research grant RG-6638 from the National Institutes of Health, Public Health Service, and from an institutional grant of the American Cancer Society, Inc., to Vanderbilt University.

[3] Part of this research was carried out in the Istituto Superiore di Sanità, Rome, Italy.

[4] Research Career Development Awardee of the U.S. Public Health Service.

GROSS ANATOMICAL EFFECTS

The effects of graded dosages of the purified protein injected into newborn mice are shown in table 1. A demonstrable biological effect was produced by the injection of 0.5 μg per 1.5 g of body weight per day, and with higher doses, there was precocious separation of the eyelids as early as 6 days after birth compared to the 12 to 13 days required in control animals. The effect of the factor on tooth eruption in newborn mice and rats is illustrated in figure 1.

TABLE 1.—Effect of injection of purified epidermal factor into newborn mice*

Dosage (μg/1.5 g/day)	Animal No.	Eyelids open (day)	Incisors erupt (day)	Weight on day 10 (g)
8	A–1	6	6	5. 5
8	A–2	6	6	5. 6
4	A–3	7	7	6. 5
4	A–4	7	7	6. 8
Control	A–5	12	10	7. 2
Control	A–6	13	10	7. 5
4	B–1	7	7	5. 4
4	B–2	8	7	5. 0
2	B–3	8	7	6. 0
2	B–4	8	8	5. 6
2	B–5	9	8	6. 0
1	B–6	9	9	6. 2
1	B–7	9	9	6. 0
Control	B–8	12	10	6. 4
Control	B–9	12	10	6. 1
1	C–1	9	10	5. 1
1	C–2	10	10	5. 6
0. 5	C–3	10	10	5. 6
0. 5	C–4	10	10	5. 6
Control	C–5	12	10	5. 5
Control	C–6	12	10	5. 7

*Newborn mice were given daily injections of 0.025 ml per 1.5 g body weight. Controls did not receive injections. Effects on litters A, B, and C are recorded.

From *Journal of Biological Chemistry* 237: 1555–1562, 1962.

PROPERTIES OF THE GROWTH FACTOR

The purity and properties of the isolated material were studied and the results are outlined. The details of the procedures have appeared in a previous publication (*3*).

Only a single component, having a sedimentation constant of $125S_{20,w}$ was detectable when the material was examined in a Spinco analytical ultracentrifuge (fig. 2).

The ultraviolet absorption spectrum showed a maximum at 277 to 278 mμ and a trough at 249 to 250 mμ. The ratio of A_{280} to A_{260} was 1.6.

The protein may be chromatographed on paper with the following solvent systems: propanol-water-ammonia and butanol-water-acetic acid. After being stained with bromophenol blue only one spot could be detected, with some tailing in the basic solvent (fig. 3). The protein band could be eluted from companion strips, and on injection into newborn mice, the lid separation and tooth eruption effects were elicited.

The results of a paper electrophoretic examination of the material are shown in figure 4. Again, only a single major component was detectable (protein stain), with some tailing in the more acid buffers. From a companion strip at *p*H 6.5, the protein band was eluted and was biologically active. From the relative positions of the glucose (fig. 4) the apparent corrected mobilities were calculated, and the results indicated that the factor had an isoelectric point at *p*H 4.2.

The factor is antigenic. Upon examination of the protein by electrophoresis and immunoelectrophoresis with cellulose acetate strips, only one electrophoretic and only one precipitating band could be demonstrated (fig. 5).

The amino acid composition of the protein after acid hydrolysis was examined, both qualitatively by two-dimensional paper chromatography and quantitatively with a Beckman automatic amino acid analyzer. All the common amino acids were present except phenylalanine and lysine. Their absence may also be considered as a criterion of purity, since it might have been expected that contaminating polypeptides would contain these amino acids. The results of the analysis are shown in table 2. The minimal molecular weight, as determined from the amino acid ratios with the assumption of 1 alanine residue per molecule, was 14,638.

That the biological activity of the factor is associated with its protein structure is also supported by the fact that this activity is destroyed by incubation with crystalline chymotrypsin or bacterial proteinase and partially inactivated with trypsin. The biological activity is stable to boiling in distilled water for 30 minutes but is destroyed when heated in 0.1 N NaOH for 1 hour or in 0.2 N HCl for 2 hours in a boiling water bath.

Finally, an antiserum against the protein may be obtained by injecting the protein together with Freund's complete adjuvant into rabbits. Incubation of the antiserum with the protein formed a precipitate and the biological activity was inhibited. The precipitate, when washed and suspended in isotonic sodium chloride, showed no biological activity when injected into newborn animals. However, when a duplicate suspension was boiled for 5 minutes to denature the antibody, between 25 and 50 percent of the original activity was recovered. These exploratory experiments indicate that the observed inhibition of biological activity was due to the presence in the antiserum of normal precipitating antibodies.

Thus it is clear that the isolated factor is a protein and that the same protein is responsible for the precocious separation of the eyelids and the eruption of the incisors. There is some evidence for the existence in salivary gland extracts of minor chromatographically separable peaks

TABLE 2.—Amino acid composition of the epidermal factor*

Amino acid	Residues per molecule	
	Calculated	Assumed
Aspartic acid	17. 30	18
Threonine	5. 06	5
Serine	11. 47	12
Glutamic acid	10. 28	10
Proline	5. 18	5
Glycine	14. 57	15
Alanine	1. 04	1
Cystine	5. 81	6
Valine	5. 16	5
Methionine	1. 68	2
Isoleucine	4. 96	5
Leucine	10. 00	10
Tyrosine	11. 07	11
Phenylalanine	<0. 02	0
Lysine	<0. 02	0
Histidine	2. 11	2
Ammonia	14. 30	14
Arginine	8. 98	9
Tryptophan	4. 61	5
Cysteic acid	Trace	
Methionine sulfoxides	Trace	
Total		127
Estimated molecular weight		14, 638

*Tryptophan was determined spectrophotometrically. The values recorded were calculated on the basis of 10 leucine residues per mole. No corrections were applied for possible destruction of serine or threonine, or for any increased ammonia formation due to destruction of amino acids.

From *Journal of Biological Chemistry* 237: 1555–1562, 1962.

with a similar biological activity, but their nature has not been ascertained. Although the injection of relatively high concentrations of the purified protein into newborn mice leads to some inhibition in weight gain and hair growth, the marked toxicity and growth-inhibiting effects (relative to the lid-tooth effects) of the crude salivary gland extracts are absent.

EPIDERMAL GROWTH RESPONSE *IN VIVO*

At this point in the investigation, the mechanisms by which the factor elicited such diverse effects were not understood. A histological study was then undertaken with Dr. George Elliott in an attempt to clarify the process. A short report of the results was published in 1963 (*4*), which provided histological evidence that the observed eyelid separation is a consequence of a more generalized biological effect, *i.e.*, an enhancement of epidermal keratinization and an increase in the over-all thickness of the epidermis.

In the first series of these experiments the protein was injected subcutaneously into newborn mice and rats for varying lengths of time. Littermates receiving injections of distilled water were used as controls. At the termination of the injection period, desired specimens were removed,

fixed, and embedded by standard procedures. Tissue sections were stained with hematoxylin and eosin.

Typical cross sections of the eyelid area from experimental and control 8-day-old rats are shown in figures 6a and 6b. In the control animal the epidermis connecting the eyelids had just started to keratinize, whereas in the experimental animal the keratinization process was considerably advanced and the eyelids had begun to separate. Sections prepared from 8-day-old mice showed an almost identical histological picture.

Whether these keratinizing changes were limited to the eyelid area or were more general was then examined. Figures 7a and 7b illustrate typical sections of back skin (epidermal portion) from 12-day-old experimental and control rats. It can be seen that in the skin of the experimental animal the thickness of both the keratin and cellular layers of the epidermis had increased. Middorsal skin sections prepared from 12-day-old mice indicated that their skin also responds to the injection of the active protein.

In a second set of experiments 12 normal mice ranging in age from 12 to 20 days received daily injections of a partially purified preparation of the factor; littermates were used as controls. After 3 to 4 weeks of injections, gross observation of the mice revealed a clearly visible increase in the diameter of the tail in all the experimental animals. Figures 8a and 8b illustrate typical sections of the epidermis of the tail in these animals. There is a marked increase in the thickness of the epidermal layers. Sections taken from the plantar surface of the hind feet of these animals showed a similar histological picture. However, in contrast to the newborn animals, no differences were noted in sections of the skin of the back from these older control and experimental animals.

Preliminary data indicate that the epithelium of the oral cavity, esophagus, and stomach of the mouse and rat can respond to the injection of the factor.

We have confirmed the histological picture by making a number of chemical measurements of pure epidermis obtained by trypsinization of standard areas of skin from 5-day-old control and experimental rats. The ratios of the average values for the experimental animals to those for the control animals were: dry weight, 1.7; total nitrogen, 1.7; deoxyribonucleic acid content, 1.3; ribonucleic acid content, 1.3; histidase activity, 1.8; and acid phosphatase activity, 1.7 (unpublished data, Angeletti, P., Salvi, M. L., Chesanow, R., and Cohen, S.).

EPIDERMAL GROWTH RESPONSE IN ORGAN CULTURE

Among the many unresolved problems presented by these data was whether the factor acts directly on the epidermis or indirectly by way of some other systemic (hormonal?) process. The techniques of tissue and organ culture seemed ideally suited for resolving this problem. The study of this aspect has been carried out at the Biochemical Section of the

Istituto Superiore di Sanità in Rome in collaboration with Dr. Rita Levi-Montalcini and Dr. Domenica Attardi. Dr. Levi-Montalcini devised the first experiments to demonstrate clearly a direct *in vitro* growth-promoting effect of the "epidermal growth factor" on embryonic chick skin.

In the following experiments we made use of a rather simple technique. Dorsal skin was dissected out from 7-day chick embryos, and fragments 1 to 2 mm in diameter were incubated in 1.2 ml of a completely synthetic medium (Eagle's basal medium) in small Falcon plastic dishes. To the experimental cultures was added 12 μg of the growth factor [Sephadex G-75, fraction as described in (*3*)]. The cultures were then placed in a humidified incubator kept at 37° C and flushed with a 4 percent CO_2-air mixture. At desired intervals the tissue was removed, fixed in Susa's fluid, and embedded by standard procedures. Tissue sections were stained with hematoxylin and eosin.

Figures 9a and 9b show sections of the control and experimental fragments of skin after cultivation for 48 hours. The epidermis of the control cultures remained almost unchanged in appearance during the period of cultivation, whereas it can be seen that a marked proliferation of the epidermal layers had occurred in the experimental culture. Photographs of a duplicate set of cultures under higher magnification are shown in figures 10a and 10b. Although a concentration of the growth factor of 10 μg per ml was routinely used in these experiments, easily observable biological effects were produced by concentrations of 0.1 μg per ml.

If the cultures are allowed to grow for 5 days, the control cultures continue to remain practically unchanged; the experimental cultures appear to keratinize (figs. 11a and 11b).

We were greatly aided in the interpretation of our results by the studies of Dr. Wessells on the cultivation of chick embryo skin (*5*). He reported that skin of 8- and 10-day embryos failed to develop in synthetic medium. Our control cultures confirm his results. The development and cornification of skin derived from 12-day chick embryos in synthetic medium were clearly demonstrated by Wessells. Our results with eyelid skin derived from the 12-day chick embryo, to be published in detail elsewhere, confirm these results and also show that in the presence of the growth factor the multiplication of the epidermal cells is more rapid and the thickness of the final cornified layer is greater.

It thus seems clear that the EGF acts directly on the skin and that its action does not necessarily involve other systemic or hormonal influences. The growth factor stimulates the division of the epidermal cells and their subsequent keratinization. Preliminary experiments have shown that the EGF can also act directly on skin explanted from the mouse embryo.

We next considered whether the dermis was necessary for the epidermal effects. In these experiments, the epidermis of the eyelid area of 11- to 12-day chick embryos was separated from the dermis by trypsinization into a sheet of cells. The epidermal sheets were then cultivated as described previously, except that Eagle's minimum essential medium was used and

the sheet of cells was placed on a fiber glass disc, the cells thus being covered only by a thin film of medium.

Figures 12a, 12b, 13a, and 13b illustrate typical sections prepared from control and experimental trypsin-separated epidermis after 48-hour cultivation. The appearance of the control epidermal cultures again was very similar to that reported by Wessells in an experiment with synthetic medium (*6*) in that ". . . the basal layer was no longer columnar and could not be identified, and as a result all epidermal cells tended to look alike in gross morphology." The absence of layering was also noted. In contrast, our experimental cultures (figs. 12b and 13b) were markedly different. Layering and the formation of pearl-like structures were clearly observed together with areas containing cells apparently undergoing keratinization. Similar results were obtained with epidermis derived from 7-day chick embryos.

This rapid apparent differentiation in pure epidermal sheets resembles the much slower differentiation observed by McLoughlin who used a very complex medium containing plasma and a chick embryo extract (*7*), which may contain traces of a specific growth factor similar to the one described here. It should be recalled that the keratinization of whole skin from very young embryos occurs in similar complex media (*8*, *9*). In our experiments the growth-stimulating effects of EGF are still marked in media supplemented with 15 percent calf serum, although the epidermal cells of the control cultures have begun to divide during the 48-hour incubation period.

Our experiments indicate that the growth factor can act directly on epidermal cells. Whether there is a stimulation of mitosis and whether the morphological changes are, in fact, analagous to normal keratinization remain unanswered. The contributions of mesenchymal tissue on the maintenance of oriented columnar cells, mitosis, and keratinization have been studied by McLoughlin (*8*) and Wessells (*6*).

DISCUSSION

We have thus isolated and characterized two specific "growth factors" from the submaxillary gland of the male mouse, the "nerve growth factor" and the "epidermal growth factor" with demonstrable specific biological effects both in the intact animal and on isolated tissue *in vitro*. Although there is evidence suggesting that the nerve growth factor plays a role in the normal life of sensory and sympathetic nerve cells, we have as yet no direct evidence that the EGF acts similarly in the normal development of the epidermis.

It is also not yet established whether these factors are synthesized in the salivary gland or elsewhere and merely stored in these organs. I have reported the failure to detect the EGF in the salivary glands of female mice and of other species and in extracts of a variety of mouse tissues (*3*). However, the *in vivo* assay was used in these experiments, and low con-

centrations of the active material would escape detection. In fact, preliminary tissue culture experiments indicate that serum may stimulate epidermal growth in tissue culture. Argyris and Argyris (*10*) and Patterson (*11*) have reported the *in vivo* growth stimulation by certain tumors on adjacent epithelial tissue. The nature of these effects must be studied further.

The effects of a number of vitamins and hormones, such as vitamin A and estrogens, on epidermal structures have been reviewed recently by Bern and Lawrence (*12*) and Laschet (*13*). The influence of hydrocortisone and vitamin A on explants of chick skin has been described by Fell (*9*), and a stimulating effect by thyroxine on such explants has been described by Wessells (*14*). The mechanisms by which these various substances, including the EGF, exert their influence on the epidermis, as well as their interrelationships, are obscure.

SUMMARY

The isolation and characterization of an epidermal growth-promoting protein from the salivary glands of mice are reviewed. The biological effects of the protein *in vivo*, on the epidermis of the mouse and rat, are described. Evidence is presented that the growth factor can act directly not only on explants of chick embryo skin, but also on dermis-free epidermal sheets.

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PLATE 3

FIGURE 1.—Photographs showing extent of incisor eruption in control and treated 8-day-old mice and rats. *A*, control mouse; *B*, mouse given daily injections of 2 μg per 1.5 g body weight of the epidermal growth factor; *C*, control rat; and *D*, rat given daily injections of 1 μg per 1.5 g body weight of the factor.

Reprinted from the *Journal of Biological Chemistry* 237: 1555–1562, 1962.

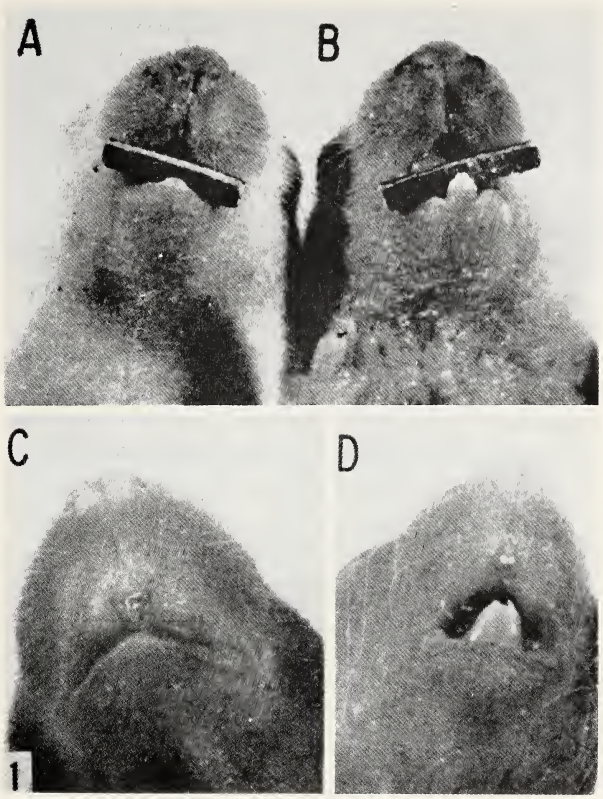

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PLATE 4

FIGURE 2.—Ultracentrifugation pattern of the epidermal growth factor at a concentration of 4 mg per ml in 0.15 M NaCl, 0.1 M Tris buffer, and 0.001 M Versene, adjusted to *p*H 7.86. The pictures were recorded at 8-minute intervals at 59.780 rpm in a valve-type synthetic-boundary cell.

FIGURE 3.—Ascending paper chromatography of the epidermal growth factor: *A*, propanol-water-ammonia; *B*, butanol-water-acetic acid. *Line* indicates starting position and *arrow* the solvent front.

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2


3
A
B

PLATE 5

FIGURE 4.—Paper electrophoresis of the epidermal growth factor. The specimens were applied at *center* line. All runs were for 16 hours at a field strength of 9 volts per cm in buffers of 0.1 ionic strength, except the barbital buffer which had an ionic strength of 0.07. Sodium acetate-acetic acid mixtures were used to prepare buffers of *p*H 3.8, 4.3, and 4.8. KH_2PO_4 for *p*H 6.5, and barbital-sodium barbital for *p*H 8.7. The electroosmotic flow is indicated by *line* representing the glucose spot, visualized in parallel strips by being sprayed with aniline hydrogen phthalate.

FIGURE 5.—Electrophoretic (*A*) and immunoelectrophoretic (*B*) patterns of the factor. Cellulose acetate strips 16 × 2.5 cm were used, with a 0.07 ionic strength barbital-sodium buffer, *p*H 8.6. The sample (20 μg of protein) was applied approximately one fourth the distance from the anode end, as indicated by *arrow*. The run was performed at room temperature for 3.5 hours at 0.5 ma per cm width of the strip. In the immunoelectrophoretic run, the diffusion under oil was allowed to proceed for 20 hours. Ponceau S was used to stain the protein.

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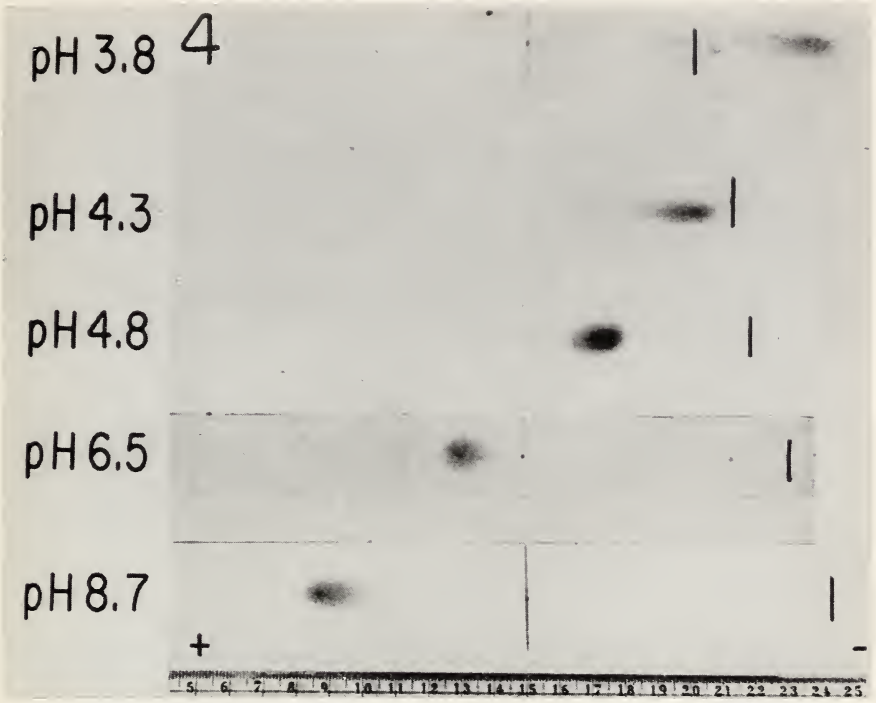
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pH 3.8
pH 4.3
pH 4.8
pH 6.5
pH 8.7
+
-

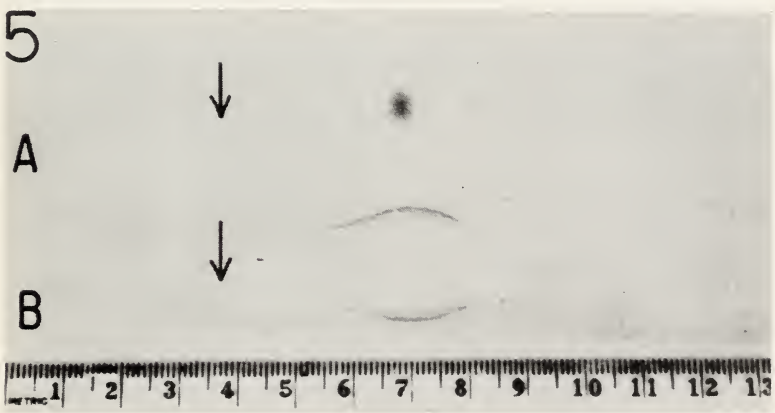
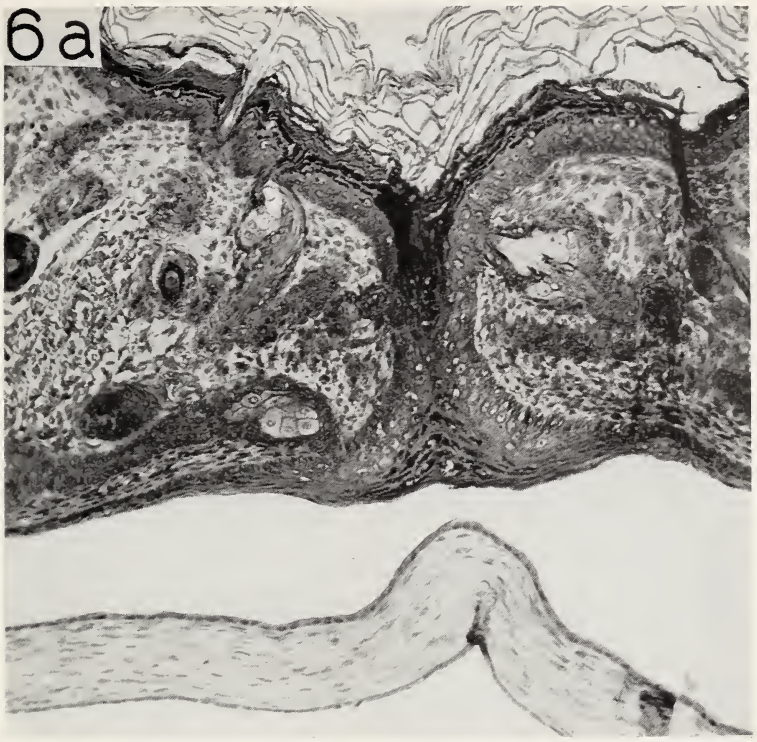

5
A
B

PLATE 6

FIGURES 6a AND 6b.—Cross sections of the eyelid area from control, *6a*, and experimental, *6b*, 8-day-old rats. Experimental animal had received daily injections (1 μg per 1 g body weight) of the epidermal growth factor. × 100

Reprinted from the *Journal of Investigative Dermatology* 40: 1–5, 1963.


6a

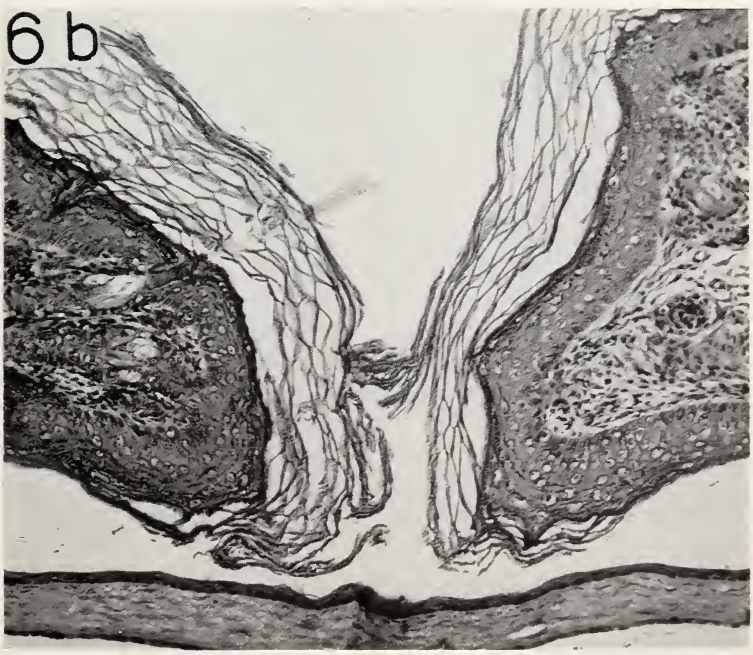

6b

Plate 7

Figures 7a and 7b.—Sections of middorsal back skin (epidermis) from control, *7a*, and experimental, *7b*, 12-day-old rats. Experimental animal had received daily injections (0.5 μg per 1 g body weight) of the active protein. × 200

Figures 8a and 8b.—Sections of epidermis of the tails of control, *8a*, and experimental, *8b*, 45-day-old mice. Experimental animal had received daily injections (8 μg per 1 g body weight) of a partially purified fraction [Sephadex G-75 fraction as described in (*3*)]. Injections were started when the animals were 13 days old. × 200

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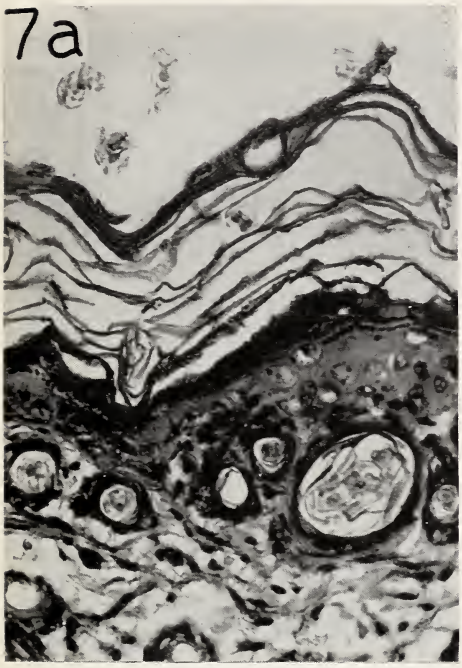
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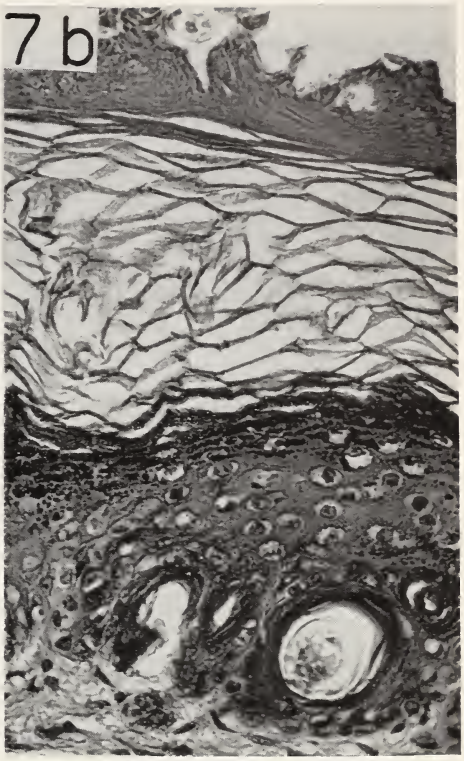
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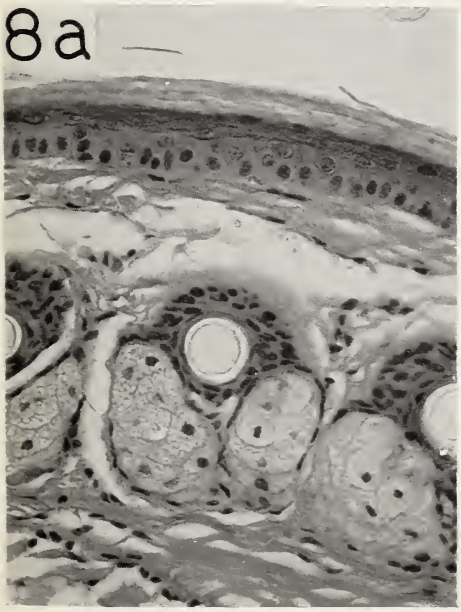
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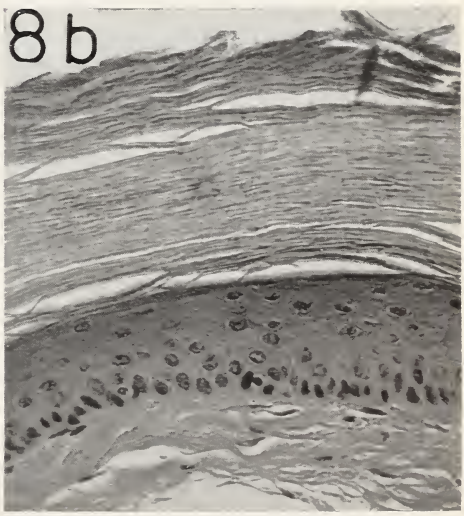
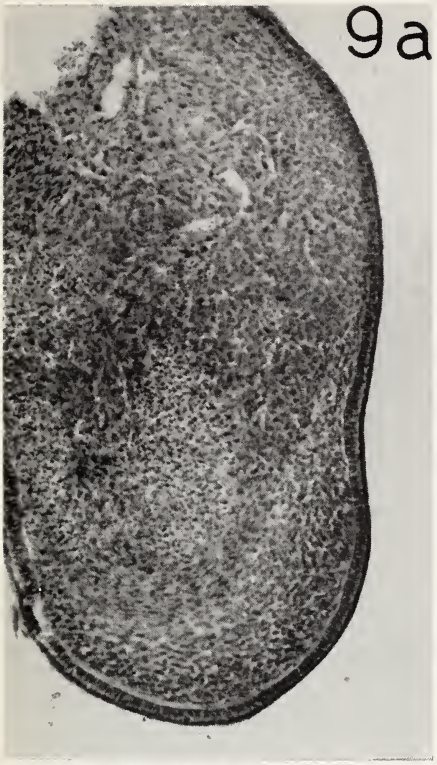

8b

PLATE 8

FIGURES 9a AND 9b.—Control, *9a*, and experimental, *9b*, explants of 7-day chick embryo skin after 48-hour incubation. × 150

FIGURES 10a AND 10b.—Control, *10a*, and experimental, *10b*, explants of 7-day chick embryo skin after 48-hour incubation. × 300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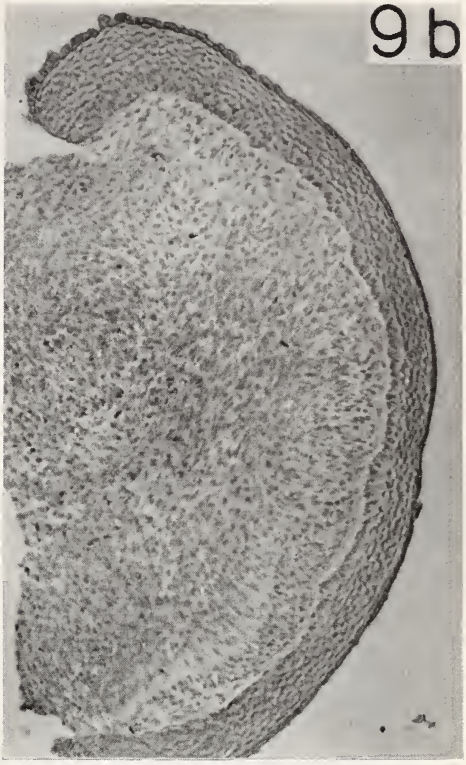
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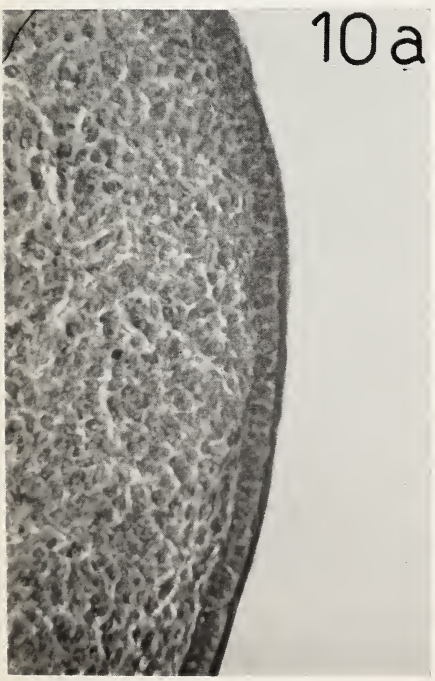
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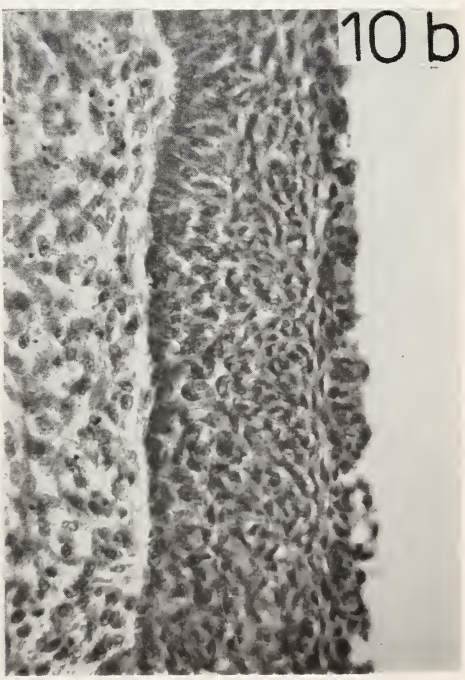
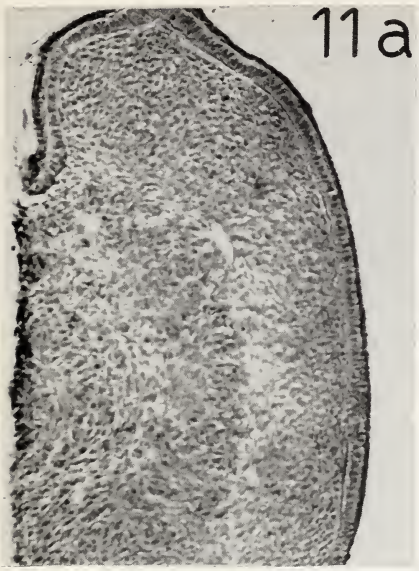

10b

PLATE 9

FIGURES 11a AND 11b.—Control, *11a*, and experimental, *11b*, explants of 7-day chick embryo skin after 5-day incubation. × 150

FIGURES 12a AND 12b.—Control, *12a*, and experimental, *12b*, explants of trypsin-separated epidermal sheets from eyelid skin of 11-day chick embryos after 48-hour incubation. × 390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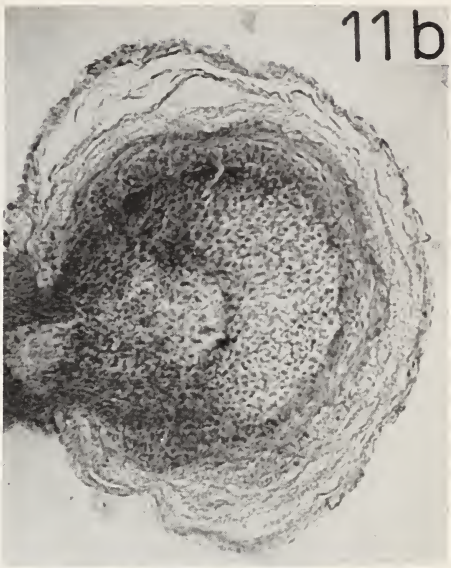
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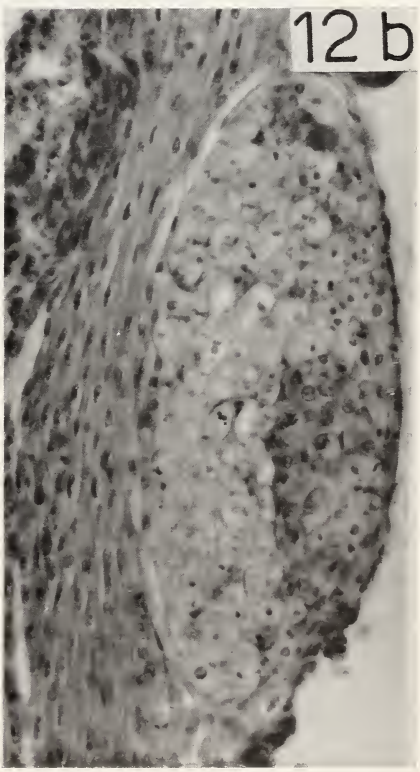
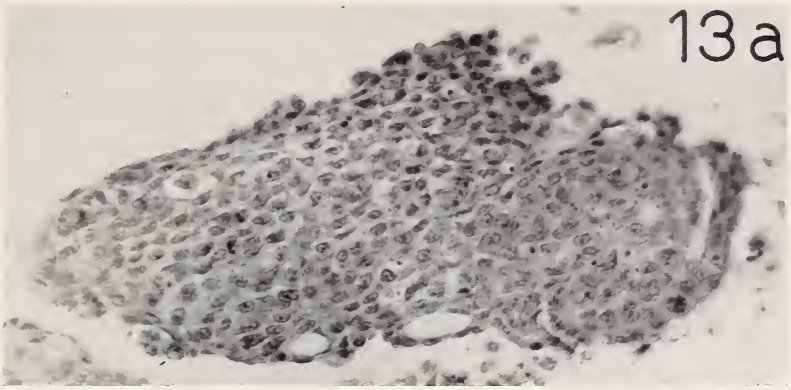

12 b

PLATE 10

FIGURES 13a AND 13b.—Control, *13a*, and experimental, *13b*, explants of trypsin-separated epidermal sheets from eyelid skin of 12-day chick embryos after 48-hour incubation. × 360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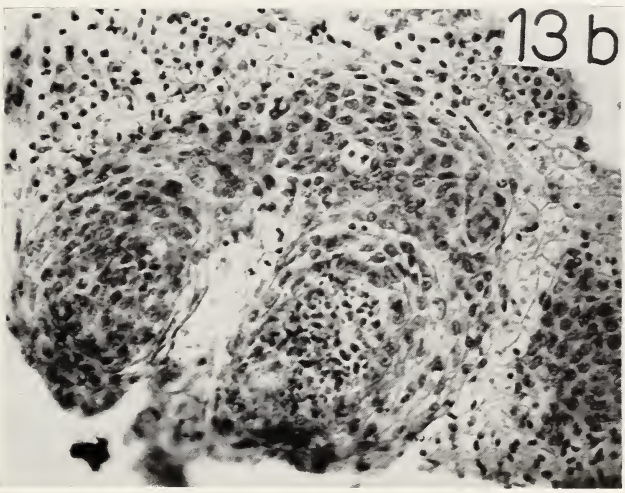
13 b

CARTILAGE INDUCTION *IN VITRO* AND SULFATE-ACTIVATING ENZYMES[1, 2]

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CHONDROGENESIS induced in explanted embryonic chick somites is becoming an increasingly favorable tool for embryologists and biochemists attempting to define the induction of cell differentiation in chemical terms. Two reasons for this interest are the relative simplicity of the tissue interactions resulting in chondrogenic differentiation and the growing knowledge of the biochemistry of chondrogenesis.

Although a few of the details may not be quite settled, there is abundant evidence that the embryonic spinal cord or notochord is capable of stimulating somites to undergo chondrogenic differentiation (*1–3*). Young embryonic chick somites do not readily form cartilage in tissue culture unless cultured in the presence of the embryonic spinal cord or notochord (text-fig. 1), or certain extracts from these tissues (*2*, *4*, *5*). In this sense the tissue interactions are simple, but undoubtedly the chemical events underlying these interactions are complicated.

One of the approaches used in the past to study the biochemical aspects of induction-differentiation has been to characterize or identify the specific chemical agents involved in the evocation of specific tissue responses. With one possible exception—the induction of mitosis in *Chlorella* (*6*)—specific chemical information pertaining to induction and differentiation has not yet resulted from these experiments. Three separate reports of the chemical induction of chondrogenesis have appeared in recent years (*2*, *4*, *5*, *7*), but the agent or agents involved have not yet been adequately described (*3*).

In this report we will discuss the problem of chondrogenic induction from a different viewpoint: the metabolic response *in vitro* of the somite cells to the chondrogenic stimulus of the notochord. For a complete understanding of the processes taking place, we must consider both the

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[2] Supported by grant HD-00380 from the National Institute of Child Health and Human Development, National Institutes of Health, Public Health Service, and National Science Foundation grant G-14123.

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[6] We are indebted to Mrs. Gladys Treon for her extraordinary assistance in preparing the large number of cultured tissues necessary for our experiments.

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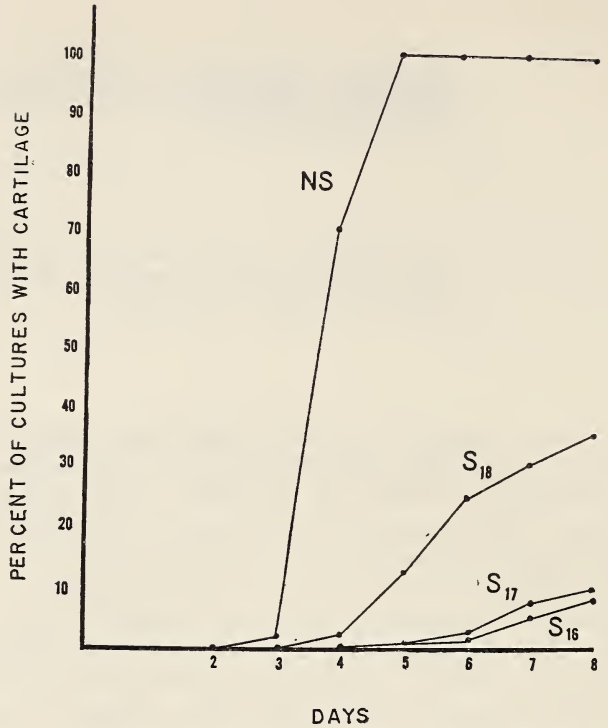


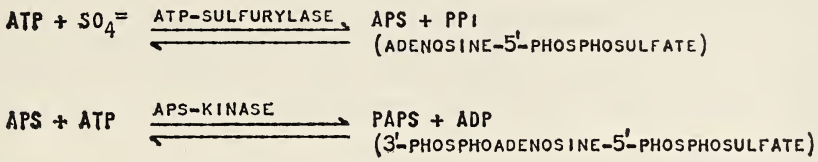
TEXT-FIGURE 1.—Percentage of cartilage formation in cultures of notochord plus somites (NS) and somites from stages 16, 17, and 18 (S_{16}, S_{17}, S_{18}). Numbers of cultures represented: NS = 200; S_{16} = 235; S_{17} = 600; S_{18} = 460. Living cultures were observed daily.

biochemistry of the inducer and, to a greater extent, the biochemistry of the responding tissue. Although we have been considering these problems for several years, we are still far from explaining the events transpiring during and after induction. However, we have some preliminary experiments pertaining to the metabolic activity of the responding tissue, the somites.

A specific metabolic response pertinent to chondrogenic tissue is the appearance and functioning of the sulfate-activating enzymes. One aspect of chondrogenesis is the sulfation of acid polysaccharides, which is effected by the transfer of sulfate from "active sulfate" [3′-phosphoadenosine-5′-phosphosulfate (PAPS) (*8*)] to a suitable acceptor molecule such as chondroitin or chondroitin sulfate (*9*). Cartilage tissue contains significant amounts of sulfated polysaccharides (chondroitin-4-sulfate, chondroitin-6-sulfate, and keratosulfate), and the sulfation of these polysaccharides is effected by the known sulfate-activating and transferring enzyme systems (*9*).

One basis for a bioassay of chondrogenic induction is consequently the ability of induced somitic tissues to form the active sulfate principle (PAPS). Assaying for the activity of the enzymes synthesizing PAPS

(*10*) can accomplish this. Text-figure 2 shows the scheme for the formation of PAPS. These are the first enzymes which have been examined in our analysis of the biochemical response of the somitic tissue to the stimulus of the notochord.



TEXT-FIGURE 2.—Scheme for the formation of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), "active sulfate."

MATERIALS AND METHODS

Preparation of tissues.—All embryos were from stages 16 and 17 [50–65-hour incubation (*11*)]. The cephalad portions of the embryos, anterior to the wing primordia, were discarded. After preliminary manual dissection (removal of embryonic membranes, limb primordia, lateral mesenchyme plate, endoderm, and mesonephros), the trunks were either used immediately for bioassay or submitted to further treatment for culture or analysis.

For further dissection, the trunks were flushed briefly in 2 percent trypsin, then rinsed in sterile balanced salt solution. After being repeatedly flushed in the rinse solutions, the epidermis separated from the trunk and was discarded. Somites and notochord were dissected from the spinal cord with a small knife. All somites were meticulously examined and cleansed of any adherent tissue, *e.g.*, mesonephros or spinal cord. Somites and notochords from 30 to 40 embryos were pooled and transferred in random samples to nutrient agar (*12*). Two types of cultures were set up: (*a*) clusters of 8 to 10 somites and (*b*) clusters of 8 to 10 somites surrounding a 0.1 mm length of notochord. One culturing session yielded 150 to 200 cultures, which were fed every 1 or 2 days with a liquid nutrient medium (*12*).

The somitic cultures are called "noninduced," since they do not readily form cartilage nodules. The "induced" cultures (somites plus notochord) form large nodules of cartilage (*see* text-fig. 1 and fig. 4). Cultures were incubated at 37° C for periods up to 8 days. Cartilage formed in induced cultures on the 4th day. Cultures of somites alone either formed no cartilage at all, or small nodules appeared late in the life of the culture. Most observations on matrix formation were visual in living cultures, but corroborative evidence was obtained on some cultures from sectioned material. Approximately 1,500 cultures of notochord plus somites (NS) and 600 cultures of somites (S) were cultured for bioassay.

Biochemical assays.—Details of the study on sulfate-activating enzymes

are published elsewhere (*13*). The following is a summary of the procedures used to detect enzymatic activity.

Assays were performed on freshly dissected and cultured tissues. Those freshly dissected were rinsed with 0.01 M phosphate buffer in 0.9 percent NaCl at *p*H 7.5.

Cultured tissues were removed from the agar with a pipette and transferred to a test tube. The tissues were then rinsed with a balanced salt solution, rinsed twice in the phosphate buffer, and ground for 3 minutes with neutral alumina and phosphate buffer at 5° C in a prechilled agate mortar.

The macerated tissue was transferred to a centrifuge tube and spun for 5 minutes at 3000 rpm (table model International Centrifuge). The supernatant was then transferred to a Spinco and centrifuged for 45 minutes at 100,000 $\times$ *g*. An aliquot of this supernatant was added to an incubation mixture containing adenosine triphosphate (ATP) (Pabst), $MgCl_2$, and $Na_2S^{35}O_4$ (Abbott Pharmaceutical, sterile solution), and incubated for 90 minutes at 37° C. Electrophoresis of the incubated sample was performed on Whatman 3MM paper in pyridine:acetic acid:water (100:10:890, *p*H 6.5). Separation of inorganic sulfate, APS, and PAPS was achieved in 70 minutes at 800 volts.

Enzymatic activity of the tissue extracts was measured by the formation of PAPS. $PAPS^{35}$ was detected by chromatogram or electropherogram scanned in a Baird-Atomic paper scanner, or an autoradiograph made of the papers with X-ray film (Kodak No-Screen Film). The film was developed in D-11 after 48 hours' exposure in the refrigerator.

RESULTS

Text-figure 1 shows the relatively long period in culture before cartilage is visible (*14, 15*). The recent report of Franco-Browder *et al.* (*16*) on the tentative characterization of chondroitin sulfate A (chondroitin-4-sulfate) in embryonic chick tissues, including somites, emphasizes the importance of the biosynthetic capabilities of these young tissues. Are they capable of synthesizing some, or all, of the macromolecules of cartilage matrix in these early stages? If they are, why is there a delay until the 3d or 4th day of culture before matrix formation becomes grossly detectable? Even with the obviation of the inducing tissues and the use of a chondrogenic extract, the delay in the onset of chondrogenesis remains (*4*).

An attempt at analysis of the early events in induced chondrogenesis is reported, with an analysis of one of the many enzyme systems required for matrix formation—the sulfate-activating enzymes responsible for the formation of "active sulfate" (PAPS). This enzyme system could conceivably be one of the last to appear in chondrogenic tissue, since most evidence indicates that the sulfation process occurs on an already polymerized polysaccharide chain (*17–20*).

PAPS Formation in 8-Day-Old Cultures

Cultures of somites plus notochord, possessing large nodules of cartilage (fig. 4), show appreciable activity with respect to the formation of PAPS (table 1). Enzyme extracts from 100 such cultures contained 200 μg of protein, and in 90 minutes' incubation formed 0.22 mμmole PAPS under our conditions. Since this is an enzyme system essential to the induction-differentiation mode under analysis, the activity was to be expected. A striking difference was seen in the activity of somites grown in the absence of notochord. Under the conditions of the assay, which could detect 0.0004 mμmole PAPS per 100 μg protein, we could not demonstrate the formation of PAPS (table 1). The cultured somites may possess these enzymes, but at a level too low to be reliably measured. Unknown sulfatase activity associated with cultured somites might also affect measured activity with respect to PAPS formation.

TABLE 1.—PAPS synthesized enzymatically by extracts of chick embryo tissues

Tissue (50–70 hr)	Experimental condition	Number of cultures per 100 μg protein	mμmole PAPS per 100 μg protein
Somites+notochord	Cultured 8 days	56	0. 11
Somites+notochord	Cultured 8 days	61	0. 11
Somites	Cultured 8 days	157	$<$0. 0004
Somites	Freshly isolated	$\simeq$700	$<$0. 003
Notochord+spinal cord	Freshly isolated	$\simeq$1, 060	$<$0. 002
Mesonephros	Freshly isolated	$\simeq$925	$<$0. 007

Sulfate-Activating Enzymes in Freshly Isolated Tissues

Tissues isolated, but not cultured, were also tested for their ability to form PAPS. Enzyme extracts from freshly isolated somites or from somites cultured for 8 days did not form detectable amounts of PAPS. Extracts of freshly isolated somites also contained less protein than a comparable amount of tissue cultured for 8 days. Somitic tissues comparable to 100 cultures produced an enzyme extract containing 14 μg of protein, compared with 70 μg in companion tissues cultured for 8 days. Even more striking is the increase in both the amount of protein present in the extract and the amount of PAPS formed if the somites are cultured with notochord. Extracts of these cultures possessed 200 μg protein per 100 cultures. It is not known whether this increase is due solely to the appearance of enzymes, or whether some of the increase is due to other proteins. Studies are in progress to determine when, in relation to visible chondrogenesis, the sulfate-activating enzymes become readily detectable.

Sulfate-Activation in Whole Trunks

In contrast to the negligible values for PAPS formation in freshly isolated or cultured somites, extracts of 32 whole trunks showed appreciable formation of PAPS (18 mμmole/100 μg enzyme protein).

The whole trunks were not trypsinized and in addition to the somites contained spinal cord, notochord, and epidermis. The epidermis is notable for its production of acid mucopolysaccharides, but we have not yet determined how much of the total enzymatic activity it contributes to the analyses of the whole trunk.

In autoradiographs of whole trunks exposed to inorganic $S^{35}O_4$, there is a noticeable incorporation of sulfate throughout the trunk, including the region of the epidermis, the notochordal sheath, part of the spinal cord, and in the somitic tissue (*21*). The pattern of sulfate fixation in autoradiographs parallels a diffusely metachromatic substance (Madden and Abbott, unpublished results). On the assumption that this represents actual incorporation and not nonselective absorption, it is in contrast to sulfate incorporation (as seen in autoradiographs) of cultured somites during the first days of culture (Madden, unpublished results). In these tissues the sulfate incorporation is negligible and no metachromasia is seen. It would seem that after dissection the somites are metabolically different, at least in sulfate fixation, from somites in the intact embryo. This raises the point of the possible artificial aspects of tissue induction-differentiation in some explant situations. Could induction in some instances be explained by the re-establishment of a previous level of specific metabolic activity (*3*)? In the chondrogenesis in explanted somites, it might be that they cannot regain their previous level without the necessary stimulus, *i.e.*, the inductive event. There is no real evidence of this yet, but the long period between induction and differentiation in explanted somites might be partly due to such a metabolic re-establishment.

CONCLUSIONS

Although sulfated polysaccharides are found in most organisms, they occur in abundance in a characteristic form in cartilage tissues. Because of their characteristic composition, sulfated polysaccharides can be used as a useful chemical index of cartilage biosynthesis (*15*).

In studies on sulfate metabolism there may be polysaccharides of varying degrees of sulfation (*22*), varying activity of sulfate-activating enzymes or sulfokinases (*19*), or even noncartilaginous sulfate molecules. Nevertheless, much useful information can be obtained from the study of sulfate metabolism in chondrogenic tissue.

Considering the long interval between tissue induction and the appearance of significant amounts of sulfated polysaccharides and cartilage matrix, an analysis of the appearance of the specific enzymes in induced somites might be more pertinent in an approach to the biochemical level of induction and differentiation than the identification of a chemical inducer. Before a specific macromolecule appears in a differentiating tissue, the requisite enzymes must be present and active. In the cartilage tissue, one of the enzyme systems must include ATP-sulfurylase and APS-kinase (text-fig. 2).

There are three related points of interest raised by the experiments on the sulfate-activating enzymes in induced chondrogenesis. One is whether the enzymes are present in what we call "noninduced" somites. In all probability, explanted somites have been exposed to inductive influences *in vivo*, but have not reached a stage of differentiation permitting chondrogenesis without further stimulus. In this sense they are induced, but the inductive event we have focused on is that stimulus which imparts the ability to form significant amounts of cartilage matrix. The chondrogenic ability of embryonic somites is drastically impaired if they are explanted to organ culture. They will, however, continue to differentiate into cartilage if supplied with appropriate stimuli. Explanted somites at zero time, or after 8 days in culture, possess very little, if any, enzymatic activity with respect to PAPS formation. This absence of enzymatic activity is understandably coincident with the absence of cartilage formation. In the presence of chondrogenic inducers, *e.g.*, notochord, the somites form significant quantities of cartilage and active sulfate (PAPS). In this sense we can say that enzymatic activity has been "induced." We are not prepared at present to say what the mechanism of increased enzymatic activity is. It is possible that the enzymes for chondrogenesis are present during all stages of development studied, but require a stimulus, *i.e.*, induction, to attain an effective level in either quantity or activity for chondrogenesis to ensue. It is equally possible, from the evidence available, that they are not present in explanted tissues until the inductive event, after which they appear as a metabolic agent. If the enzymes are "induced," then the absence of activity in cultured (noninduced) somites is explicable.

If the enzymes could be found in freshly isolated, nontrypsinized somites, the lack of activity in cultured somites might be due to the fact that they were removed from the inductive continuum of the embryo and either regressed to a lower level of activity, or maintained a level too low for subsequent differentiation (*3*). It is also possible that the somites, if not forming cartilage, produced sulfatases.

The possible role of degradative enzymes in directing differentiation is one that has received little attention. It is only speculation, but this could be a controlling factor in tissue differentiation, since the embryonic somite is a heterogeneous mass of cells and differentiates into other tissues, notably muscle. Cells can be prevented from forming cartilage by specific enzymes as well as stimulated by specific enzymes. Not to be overlooked in these speculations is the fact that cartilage tissue is chemically heterogeneous within itself and structurally heterogeneous within the organism.

Although we could not detect sulfate-activating enzymes in trypsinized, isolated somites, the possibility remains that they are present *in vivo*. This could be inferred from analyses by others (*16*, *21*).

There is probably no one enzyme system or inductive event responsible for chondrogenesis. The complexed protein and associated collagen are equally important in relation to inductive events and tissue differentiation.

Possibly the most intriguing question relating to these experiments is the apparent inconsistency between enzymatic assays on precultured tissues, *i.e.*, undissected trunks containing skin, somites, spinal cord, and notochord, and tissues either prepared for culture or actually cultured. The precultured tissues showed a higher level of PAPS formation than somites cultured without notochord. This raises various possibilities. One is that in the intact trunks there may be more than one sulfokinase system. It cannot be determined from our analyses whether we are assaying the activity of one or more sulfate-activating enzyme systems. This might be the reason for the difference in the activity of intact trunks and cartilage-forming somite cultures. The enzymatic activity of whole trunks may be associated wholly or partly with the skin, which is known to metabolize sulfate and produce sulfated polysaccharides. Another possibility is that during preparation for culture, with the proteolytic digestion and manual dissection of the tissues, the enzymatic activity falls to a level insufficient for continued matrix synthesis.

These speculations stress the difficulty of attempts to explain, from a few simple experiments, the complicated series of interrelated events occurring in tissue induction and differentiation. What the embryologist calls "embryonic induction" is a series of continuing events, and it is difficult at our present stage of knowledge to define the most important of these events in discrete chemical terms. Although chemical agents have simulated tissue induction, the chemistry of induction is still obscure. We believe that only by following the chemistry of the responding tissue can a closer approximation to an understanding of tissue induction and differentiation be attained. Before attempts are made to place a specific chemical into a pattern of specific tissue response, it would be well first to define the pattern.

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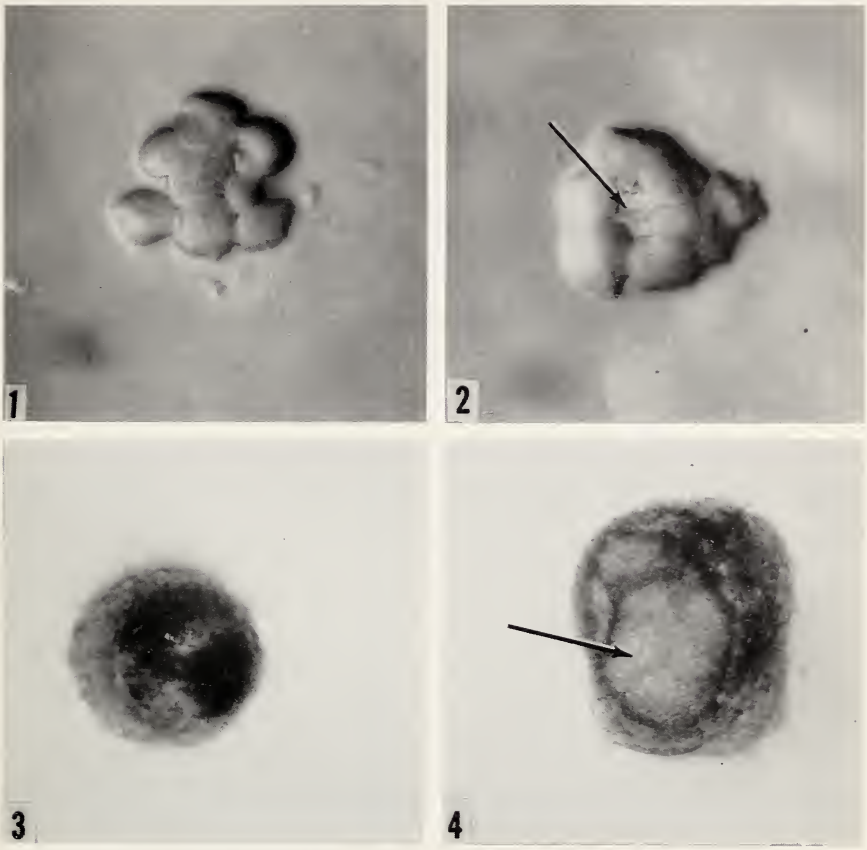
Plate 11

Figure 1.—Cluster of freshly isolated somites (stage 16) placed on nutrient agar × 16

Figure 2.—Cluster of freshly isolated somites (stage 16) surrounding piece of notochord (*arrow*) placed on nutrient agar. × 16

Figure 3.—Culture shown in figure 3, 8 days later. No visible cartilage in culture × 16

Figure 4.—Same culture shown in figure 2, showing prominent nodule of cartilage (*arrow*). Living culture, 8 days old. × 16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CONTROL OF SPECIFIC SYNTHESIS IN THE DEVELOPING PANCREAS[1]

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EMBRYONIC differentiation includes the selective control of cell proliferation and the expression of diversified morphology and physiological function. The chemical mechanisms of regulation of such complicated processes may be exceedingly varied but, at least at present, they all share the common feature of being unknown. Many basic principles of development at the cell and tissue level, however, have been defined; for example, there is considerable evidence to suggest that distinctive cell types frequently develop from an interaction between dissimilar precursor tissues. As summarized in table 1, a number of organ systems arise from an association of mesenchyme, a tissue composed primarily of fibroblast-like cells that characteristically produce an extracellular matrix of collagen and other materials, and epithelium, a highly ordered, closely packed system of cells with little extracellular material. The results of experiments *in vivo* and *in vitro* have supported the concept of an epithelium-mesenchymal interaction. Experiments *in vivo*, involving removal or transposition of the interacting elements or the interposition of a suitable barrier between them, have been intrinsically informative, but the major experimental evidence defining the interaction has been derived from experiments *in vitro* involving separation and recombination of the tissue elements. The morphological features of differentiation are produced only in the presence of both tissue components. The demonstration of morphogenesis of organ rudiments *in vitro* is especially significant from an experimental point of view, since it implies that at least some phases of cytodifferentiation can be obtained *in vitro* and, thus, are generally susceptible to more searching kinds of analyses. The further demonstration of an interaction of the tissues across a filter membrane under conditions *in vitro* (*6*, *7*, *14*, *21*), for example, indicated that direct cell contact among the tissues was not necessary but that diffusible or at least mobile substances were causally involved.

Of the various possibilities for an analysis at the molecular level of tissue interactions leading to cytodifferentiation, the pancreas system was

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[2] John Simon Guggenheim Memorial Fellow, 1963–64.

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TABLE 1.—Epithelium-mesenchyme interactions in embryonic development

Organ	*In vivo* removal, transposition, or interference		*In vitro*		
			Separation and direct recombination	Separation and recombination transfilter	
Kidney	+	(*1*)	+	+	(*2–4*)
Salivary			+	+	(*5, 6*)
Thymus			+	+	(*7*)
Pancreas			+	+	(*8*)
Thyroid			+		(*9*)
Pituitary			+		(*10*)
Mammary			+		(*11*)
Lung			+		(*12*)
Skin	+	(*13*)	+	+	(*15, 16*)
Feather	+	(*17*)	+	−	(*18, 19*)
Limb bud	+	(*20–23*)			

chosen because, in addition to distinctive morphological changes, differentiation in this tissue leads to the production of a number of chemically well-characterized enzymes and hormones. The appearance of these substances, therefore, could be used to quantitatively assess the differentiation process.

Golosow and Grobstein (*8*) have already defined some pertinent developmental features of this system: 1) Morphogenesis of pancreatic epithelium occurs *in vitro* if mesenchymal tissue is present; 2) the tissue interaction can occur across a filter membrane; and 3) a variety of mesenchymal tissues are effective in supporting the morphogenesis (in contrast to some systems in which there is apparently a more specific requirement for a particular mesenchymal tissue). The experimental system of Golosow and Grobstein (*8*), employed in the present study, is schematically presented in figure 1. The pancreatic rudiment was dissected from an 11-day mouse and separated into mesenchyme and epithelial portions by appropriate incubation in trypsin solution and subsequent mechanical agitation with a micropipette. The epithelial rudiment was then quartered into sections, each containing approximately 1,500 cells (*22*), and each section placed on a separate filter assembly by means of a plasma clot. The mesenchyme was dissected from another pancreatic rudiment, or more usually from the salivary rudiment, of a 13-day mouse embryo and placed on top of the filter. The assembly was then put in the well of a suitable culture dish containing the basal medium and incubated at 38° C. The appearance of typical cultures during incubation is shown at the bottom of figure 1. Fell (*22*), in this laboratory, showed that the cell number increased about 30 times during a 5-day culture period, but proliferation was most rapid during the 2d and 3d day. During this period the cells organized into acinar clusters that could be recognized visually in living epithelia as small doughnut-like structures. At the end of the 4th day or beginning of the 5th, the cultures became optically opaque, presumably due to the intracellular accumulation of the specific pancreatic products. In the system without mesenchyme,

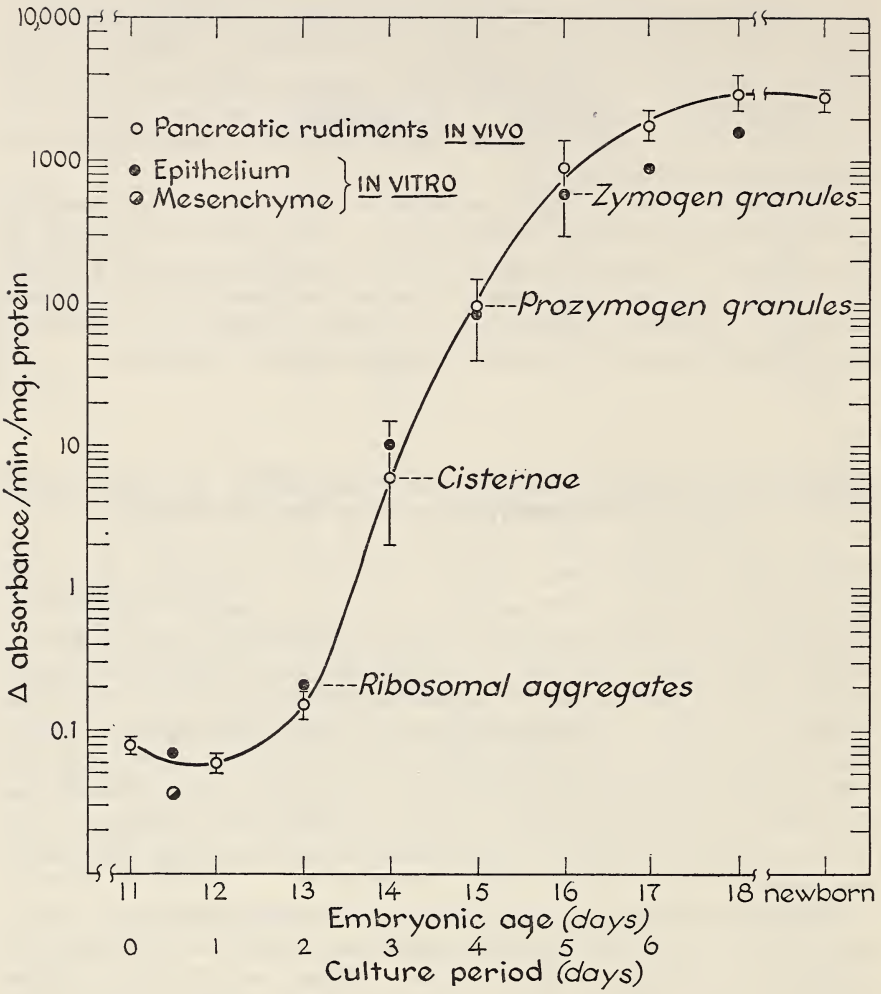
the epithelium spreads, usually without developing a perceptible morphological pattern and certainly without the formation of optically dense regions. The rate of cell proliferation was also significantly lower in the absence of mesenchyme (*24, 25*). This system, therefore, involves the regulation of cell duplication as well as specific morphological and presumably metabolic events.

The present work primarily concerns the process of development of specific metabolic function in this system. The aim has been, first, to characterize the kinetics of appearance of a specific pancreatic product, the enzyme amylase; and second, to employ amylase activity measurements as a quantitative indication of functional differentiation to define some characteristics of the epithelium-mesenchymal interaction.

AMYLASE ACTIVITY DURING DEVELOPMENT OF THE PANCREATIC EPITHELIUM *IN VIVO* AND *IN VITRO*

The enzyme amylase was chosen for the present studies because it is a prominent product of the pancreas and can be assayed conveniently. The classical amylase assay, involving the disappearance of the starch-iodine chromophore, was readily modified (*23*) to an appropriate degree of sensitivity (approximately 10^6 enzyme molecules, assuming the turnover number of the mouse and hog pancreas enzymes is the same). Protein was measured by a microadaptation (*23*) of the Folin-Lowry method (*26*), sensitive to about 0.2 μg protein. All the experimental results are expressed in terms of specific activity; thus, differences reflect changes in the proportion of this enzyme activity relative to other proteins in the system.

Text-figure 1 shows the specific activity of amylase in pancreatic rudiments isolated from mice embryos at various ages and also of the pancreatic epithelia at various periods of culture. Eleven- and 12-day rudiments exhibited a low level of activity (less than 10 molecules of amylase/cell, again assuming the turnover number of the mouse pancreas enzyme is the same as the hog pancreas enzyme). In rudiments from 13-day embryos there was a significant increase in specific amylase activity and in subsequent days it increased to about 50,000-fold over the basal level. The specific activity of amylase in cultures followed closely that of the directly isolated rudiments, at least during the first 5 days in culture. After longer periods there was greater variability in the activity of individual cultures and some central necrosis was often observed, perhaps as a result of the release and "activation" of hydrolytic enzymes. A major source of variation in analyses *in vivo* and *in vitro* was the uncertainty of the age of the embryos ($\pm$ 12 hours), but the generally satisfactory correlation in the development of amylase activity in the two systems with respect to both time course and quantitative amylase accumulation suggested that the *in vitro* system is an adequate reflection of the *in vivo* phenomenon.



TEXT-FIGURE 1.—Amylase activity in the developing pancreas and correlation with intracellular structures. *Open circles* represent the specific activity of intact pancreatic rudiments (epithelium + mesenchyme) dissected from embryos. *Points* represent averages of approximately 40 rudiments in early periods and steadily decrease to about 20 at later embyronic stages. *Solid bars* indicate the range of values in the experimental sample. *Closed circles* present the average specific amylase activity of *in vitro* pancreatic epithelia cultures at various intervals. At the early times more than 75 cultures were included in these analyses. At later culture periods this number decreased to 30. The dispersion about the average value was similar to that found in the intact rudiment. *Half-closed circles* represent amylase specific activity of salivary mesenchyme used in the experiments (24 mesenchymal tissues). Kallman's observations (*27*) of the approximate time of the appearance of intracellular structures, robosomal aggregates, cisternae, prozymogen granules, and zymogen granules are indicated by *dotted lines*.

The specific amylase activity in mesenchyme tissue does not change appreciably during the culture period, but, if environmental influences are causally related to the changes observed in the pancreatic epithelium, they are without effect on mesenchyme cells.

The data presented here complement other studies on the developing pancreas. Kallman (*27*), with the electron microscope, observed the fine structure of cells during the process of pancreatic morphogenesis, especially in the *in vitro* culture system. Abundant ribosomal aggregates were seen throughout the initial 2 days of culture. In view of the recent studies in reticulocytes, these might be the functional units of protein synthesis (*28*, *29*). At approximately the time of initial rise in amylase activity, presumably indicating an increase in amylase synthesis, isolated rough membranes (cisternae) were detected in a few cells. The number and size of the cisternae increased in a more or less parallel fashion with the increase in specific activity of amylase during continued cultivation. In the later periods of culture, prozymogen granules containing relatively little material were observed, and gradually the heavily packed zymogen granules accumulated. The zymogen granules, no doubt, are largely responsible for the opacity of differentiated epithelial cultures. These combined studies suggest a smoothly coordinated sequence of cytological events in which synthesis of amylase, and probably other pancreatic enzymes, is correlated with the formation of intracellular structures that are probably involved in their synthesis storage and secretion.

Wessells (*30*) has shown by a combination of autoradiographic and cytological methods that the cell proliferation occurring throughout the culture period is primarily localized in the outer regions of the tissue; differentiation, on the other hand, proceeds in the internal areas where there are few, if any, mitoses and little DNA turnover. The kinetics of amylase increase presented in figure 1, therefore, is not a reflection of the synchronous differentiation of a homogeneous population of cells, but represents the integrated activities of cells in different stages of differentiation. Rather than the time scale on the figure, therefore, as little as 2 days may be required from the initiation of specific synthesis in a particular cell until it is packed with zymogen granules. If the increase in amylase activity is a result of synthesis of new enzyme molecules, and if the turnover number of a single protein synthesizing unit is approximately one protein molecule per minute (*24*, *25*), then the number of enzyme molecules produced per cell (employing the turnover number of the hog pancreas enzyme) during the period of amylase accumulation (3–5 days of culture) would require about 20 to 40 amylase synthesizing units per cell. Because only a limited population of the cells is involved in zymogen granule formation (*30*) and because the time of synthesis of amylase may be several minutes, the number of amylase synthesizing units may be higher by as much as an order of magnitude in the cells involved in the synthesis of this protein.

TABLE 2.—Mesenchymal competence for supporting pancreatic epithelial differentiation

Organ	Morphogenesis*	Amylase specific activity (Δ absorbance/min/mg protein)
Salivary	+	800
Pancreas	+	240
Kidney	+	—
Lung	+	—
Stomach	+	—
Spleen	+	300
General mesenchyme	+	—

*In part from Golosow and Grobstein (8).

REPLACEMENT OF MESENCHYME REQUIREMENT FOR PANCREATIC EPITHELIUM DIFFERENTIATION BY EMBRYO PARTICLE FRACTION

In subsequent experiments the level of specific amylase activity was used as a quantitative indication of differentiation. These measurements were also correlated with visual observations of optically dense areas, which presumably reflect the presence of zymogen granules and are represented by positive (+) morphogenesis.

Attempts to simplify the differentiative system were in part based on the finding, illustrated by the data in table 2, that a variety of mesenchymal tissues are competent to support morphogenesis of pancreatic epithelium. The embryo, it was reasoned, was so sufficiently enriched with mesenchymal tissues that an extract of the whole embryo might be effective in replacing intact mesenchyme in this system. The results of an experiment designed to test the action of a crude extract of embryo in this system are reported in table 3. In the absence of embryo extract, no differentiation was observed. In confirmation of previous studies,

TABLE 3.—Requirements for *in vitro* differentiation of pancreatic epithelium

Experiment	Culture conditions		Analysis 5-day cultures		
	Mesenchyme	Chicken embryo* extract (%)	Morphogenesis†	Amylase specific activity (Δ absorbance/min/mg protein)	
				Mesenchyme	Epithelium
1	+	0	—	None detected (<0.2)	4
2	+	3	++		250
3	+	20	++		220
4	—	3	—		None detected (<0.5)
5	—	10	++		165
6	—	20	++		240

*Concentration based on volumes of crude embryo extract [embryos extracted with an equal volume of Tyrode's solution under defined conditions (23)].

†Microscopic evidence of zymogen granules in cultures

the presence of 3 percent embryo extract supported differentiation in the presence, but not in the absence, of mesenchyme. Higher levels of embryo extract did not apparently affect the system in the presence of mesenchyme, but supported cytodifferentiation in the absence of added mesenchyme. With high levels of embryo extract, the cutures resembled those obtained with added mesenchyme, both in general size and amylase specific activity. Hence the embryo extract replaced the significant features of the mesenchyme effect in these cultures.

The results of assays for the factor or factors supporting pancreatic development in fractions from embryo extract are shown in table 4. The activity was broadly distributed within the sedimentable fractions and was virtually absent from the "soluble" fractions. Various methods of extraction in both balanced-salt (Tyrode's solution) and sucrose media have all given fractions in which the biological activity is broadly distributed. The "differentiative activity" in the fractions, based on protein or mass, was higher in the fraction sedimenting between 10,000 and 100,000 $\times$ *g*, but this may be misleading since there is some evidence suggesting that the observed activity is influenced by the state of aggregation of the material; we have thus hesitated to express the "differentiative activity" in terms of specific activity.

As shown in table 5, adult liver microsomal and mitochondria fractions prepared by standard procedures, as well as DNA and RNA, were all inactive when tested over a wide range of concentrations. In addition, collagen, which is produced more or less specifically by mesenchymal tissues, was also inactive over a wide range of concentrations.

Results of studies on the effect of some hydrolytic enzymes on the activity of the active fractions are shown in table 6. Ribonuclease- and deoxyribonuclease-treated particle fractions still retained their biological activity under conditions in which the respective substrates were hydrolyzed rapidly. These experiments appear to rule out soluble nucleic acids as the dominant factors in this system, but, of course, do not rule out forms of nucleic acids unavailable to the enzymes. Treatment with trypsin, on the other hand, destroys the activity in the fractions. The

TABLE 4.—Differentiative activity of embryo extract fractions

Experiment	Fraction tested at 20 percent level*	Analysis 5-day cultures: Morphogenesis	Analysis 5-day cultures: Amylase specific activity (Δ absorbance/min/mg protein)
1	Initial extract	++	370
2	1000 $\times$ *g* Sediment	++	300
3	1000–10,000 $\times$ *g* Sediment	++	235
4	10,000–100,000 $\times$ *g* Sediment	++	330
5	100,000 $\times$ *g* Supernatant	−	2
6	Ultrafiltrate	−	$<$0. 5
7	10,000–100,000 $\times$ *g* Sediment + 100,000 $\times$ *g* Supernatant	+	130

*Based on volume of fractions (sedimentable, suspended in 0.3 initial extract volume).

TABLE 5.—Effect of cell fractions and specific macromolecules on differentiation of pancreatic epithelium

Experiment	Additions to:	Concentration*	Analysis 5-day cultures: Morphogenesis	Analysis 5-day cultures: Amylase specific activity (Δ absorbance/min/mg protein)
1	Adult liver microsomal fraction	0.4–20%*†	–	None detected
2	Adult liver mitochondrial fraction	1–20%*†	–	(<0. 5)
3	DNA (salmon sperm)	10–1000 μg/ml*	–	(<0. 5)
4	RNA (yeast)	10–1000 μg/ml*	–	(<0. 5)
5	Collagen (calf skin)	20–4000 μg/ml*	–	(<0. 5)
6	Embryonic 100,000 × *g* sediment	20%‡	++	540

*At high concentrations cultures became necrotic.
†Based on volumes of cell fractions obtained from a 1 : 10 homogenate according to conventional procedures.
‡Based on volumes of 10–100,000 × *g* sediment suspended in 0.3 volume original extract of embryo extract.

partial loss of activity in fractions treated with trypsin and the trypsin inhibitor suggests that the trypsin inhibitor does not completely inhibit the enzyme under these conditions, since incubation in the presence of inhibitor alone does not significantly change the activity over controls. These data suggest that protein may play a dominant role in the biological effect, but in this complex system secondary effects cannot be ruled out. The fact that the material is found in embryonic cells, but not in adult tissues so far tested, suggests that the active material is primarily concentrated in embryonic tissues. The general characteristics of the particle fractions suggest the differentiative activity may reside in the intracellular matrix or may be bound to the cell membrane. The questions of: 1) the possible multiplicity of active factors and 2) whether the action of mesenchyme and embryo fractions are mediated by the same molecules may be resolved by isolation and characterization of the active substance(s).

TABLE 6.—Effect of hydrolytic enzymes on differentiative activity of embryo particle fraction

Experiment	Treatment of 100,000 × *g* particle fraction (min)*	Analysis 5-day cultures: Morphogenesis	Analysis 5-day cultures: Amylase specific activity (Δ absorbance/min/mg protein)
1	None	++	480
2	Ribonuclease, 60	++	500
3	Deoxyribonuclease, 60	++	480
4	Trypsin, 15	+	40
5	Trypsin, 60	–	0. 5
6	Trypsin + trypsin inhibitor, 60	++	100
7	Trypsin inhibitor, 60	++	560

* All incubations were carried out at 38° C.

EFFECT OF ACTINOMYCIN D ON THE DIFFERENTIATION OF PANCREATIC EPITHELIUM

Some initial experiments on the nature of the differentiative process have centered about the events during the early period of development of pancreatic epithelia *in vitro*. It is considered significant that the system becomes independent of mesenchyme and embryo fractions about the time of the initial rise in amylase activity. This correlation suggests that the action of the particle fraction may be to initiate or support a series of events culminating in the development of an effective synthesizing system for the specific pancreatic enzymes. An approach to the examination of this postulate has employed specific inhibitors. Inhibition of specific protein synthesis by actinomycin D may reflect a requirement for DNA-dependent RNA synthesis (*31*, *32*) during the period of observation. Preliminary experiments have shown that treatment of the pancreatic epithelia with 1 μg per ml of actinomycin D, a concentration usually used for inhibition of RNA synthesis, resulted in loss of viability of the cultures. But at a concentration of 0.01 μg per ml inhibition of growth was observed, though the cells appeared healthy and mitotic activity was observed. The results of some of the experiments testing the effect of this low level of actinomycin D on differentiation of pancreatic epithelium in the presence of mesenchyme and embryo particle fractions are summarized in figure 2. Cultures were treated with actinomycin D for a 5-hour period at various times of culture. The cultures were then incubated for the remainder of the 5-day period. There is a gradient of sensitivity of the cultures according to the period of treatment, *e.g.*, when cultures are treated from 48 to 53 hours, the tissues grow but produce only a few zymogen granules. The specific activity of amylase is far below that normally found in 5-day cultures but is more than in normal 48- to 53-hour cultures; therefore, there is apparently a limited continued synthesis of amylase under these conditions. On the other hand, at 72 hours the system is more refractory to actinomycin D; the subsequent synthesis of amylase and the visual evidence of zymogen granule formation approach that of untreated cultures. Prolonged cultivation of the tissues does not appreciably alter these results. Thus the data are consistent with the postulate that a relatively long-lived messenger RNA, necessary for the synthesis of specific pancreas enzymes, is synthesized from 48 to 72 hours in the cultures of these experiments. The generally similar results in the experiments in which the active particulate fraction from the embryo replaced the mesenchyme confirm the general similarity of the action of this fraction and that of the mesenchyme.

Pretreatment of mesenchyme with as much as 1 μg per ml actinomycin D did not apparently affect the viability of these cells or the ability to support the differentiation of the pancreatic epithelium. This suggests the active product may perhaps be synthesized by the cells routinely and is not produced in response to the addition of epithelium. The data are suggestive of an action by the active particulate fraction and mesenchyme

on the genome of the epithelial cell, which makes it available for specific RNA synthesis.

Action of the embryo fraction on the pancreatic system cannot be enzyme induction in its simplest form: There is an effect on the growth and morphogenesis of the cells within the culture before the synthesis of messenger RNA for amylase; moreover, there is a considerable period after addition of the particles before specific synthesis of amylase begins. Indeed, it is not claimed that the mesenchyme or the active particulate fractions confer an essential "pancreatic character" on the epithelium. The pancreatic epithelium obtained from 11-day mouse embryos already may be biased in the direction of pancreas formation. The possibility that these particulate fractions instead may play a more general role in supporting the differentiation of a number of epithelia must be considered. It is thus possible that the discovery of the particulate fractions is of more relevance to the problem of obtaining differentiative behavior under conditions *in vitro* than to one of classical embryonic induction. What is perhaps most significant from all of this is that this experimental system appears to be willing to tell us more.

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PLATE 12

FIGURE 1.—Schematic representation of experimental procedure for pancreatic morphogenesis *in vitro*.

The experimental procedure involves: 1) dissection and separation of embryonic tissues (*first line* of photographs); 2) appropriate placement of tissues on filter assembly: pieces of pancreatic epithelium on underside of the filter in a plasma clot, the mesenchyme stranded on upper side of the filter; and 3) incubation of filter assemblies under defined nutritional conditions, at 38° C, 5 percent CO_2 and air.

Solid line below each tissue indicates 150 μ. Internal diameter of the filter assembly is 3.3 mm. The relative size on filter assembly of the mesenchyme and epithelium is overemphasized by approximately twofold and tenfold, respectively. The appearance of typical pancreatic epithelia after various periods of culture is indicated by photographs at *bottom* of figure. The camera was focused on the underside of the filter directly on the epithelial tissue. The mesenchyme spread diffusely on the upper filter membrane contributes to the background.

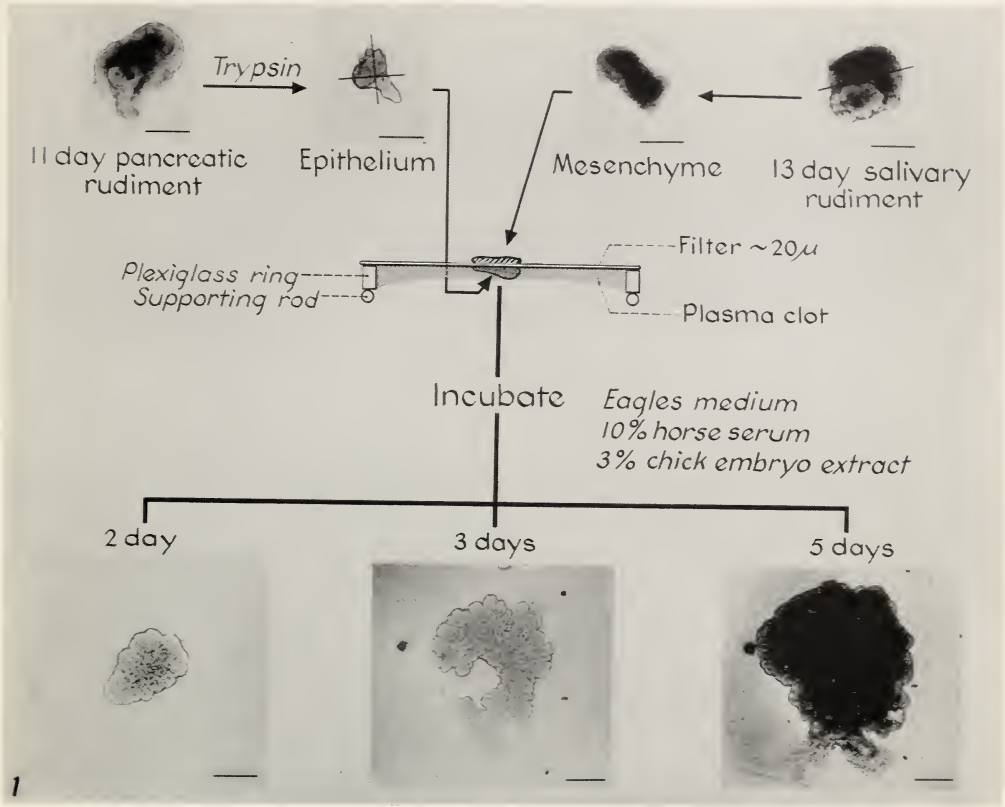
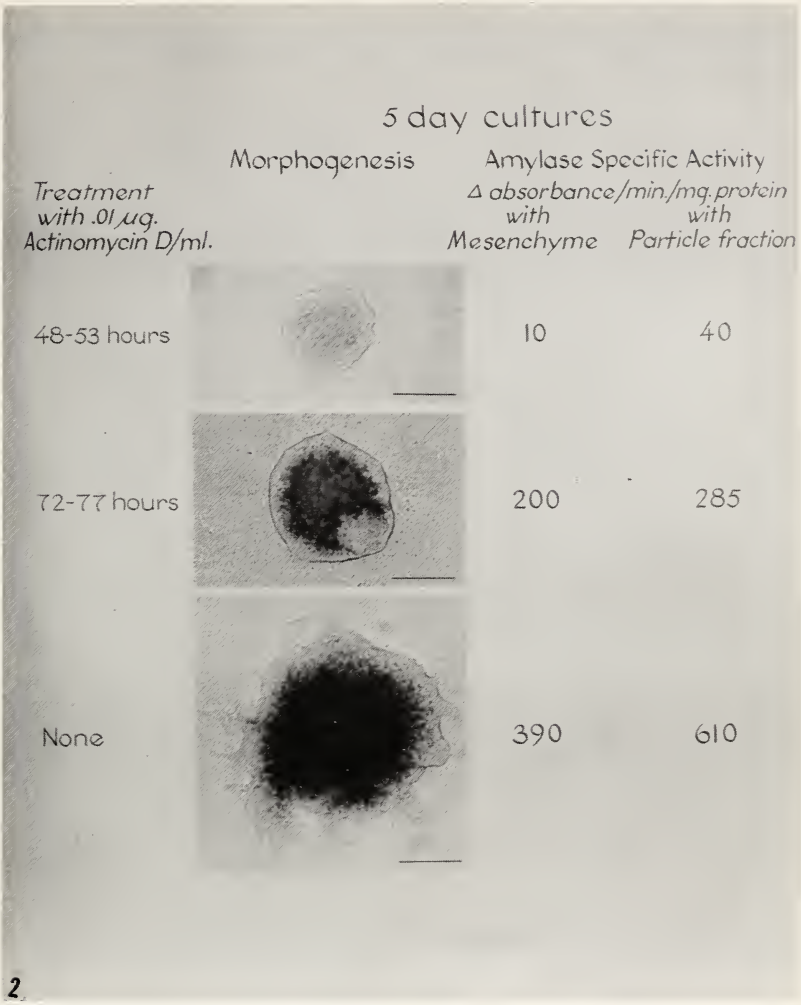

Trypsin
11 day pancreatic rudiment
Epithelium
Mesenchyme
13 day salivary rudiment
Plexiglass ring
Supporting rod
Filter ~20μ
Plasma clot
Incubate
Eagles medium
10% horse serum
3% chick embryo extract
2 day
3 days
5 days
1

PLATE 13

FIGURE 2.—Effect of actinomycin D on differentiation of pancreatic epithelium.

Pancreatic epithelia cultures in the presence of mesenchyme or the 10,000–100,000 $\times$ *g* sediment fraction from 9-day chicken embryo extract (suspended in culture medium) were treated with actinomycin D for periods indicated. Afterward cultures were washed 3 times with Tyrode's solution and incubated with standard culture medium (with embryo particle fraction where appropriate) until 120 hours total culture period (media were changed at 24-hour intervals). Morphogenesis is indicated by photographs of typical cultures at 120 hours. *Solid line below* cultures indicates 200 μ.

The amylase specific activities are average analysis of approximately 40 cultures (treatment 48–53 hours), of approximately 30 cultures (treatment 72–77 hours), and approximately 30 cultures (no treatment).


5 day cultures
Morphogenesis
Amylase Specific Activity
Δ absorbance/min./mg. protein
Treatment with .01 μg. Actinomycin D/ml.
with Mesenchyme
with Particle fraction
48-53 hours
10
40
72-77 hours
200
285
None
390
610
2

STUDIES ON THE HORMONE-INDUCED DIFFERENTIATION OF MELANOBLASTS INTO MELANOCYTES IN EXPLANTS FROM XANTHIC GOLDFISH TAILFIN[1, 2, 3]

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In this paper we report some of our recent results on hormone-induced melanocyte formation in xanthic goldfish. This work originated from the studies with normal and hypophysectomized xanthic goldfish by Chavin in the early 1950's. It was observed that, whereas normal xanthic goldfish turned partially black as a result of physiological stress, hypophysectomized xanthic goldfish did not (*1*).

Working with primary explants of xanthic goldfish tailfin, Hu and Chavin demonstrated a direct effect of hormone on melanization (*2*). Several hormone preparations—adrenocorticotropin (ACTH), melanin-stimulating hormone (α-MSH), β-MSH, and prolactin—were tested, but the variations in purity and activity toward melanization of the various preparations, in conjunction with the failure of a crude "intermedin" preparation to induce melanization *in vivo* (*1*), made it difficult to decide which of these hormones is responsible for the observed formation of melanocytes.

Later work revealed that stress-induced melanization is preceded by an increase in the tyrosinase activity in the skin (*3*, *4*). Further, it was demonstrated that hormone-induced melanocyte formation is inhibited by colchicine (*5*, *6*), a well-known inhibitor of mitosis (*7*).

We have now extended certain aspects of this investigation. The results discussed in the following sections vary from fairly complete and definitive to quite incomplete and exploratory. The latter are included because they are helpful in the formulation of a working hypothesis which serves as a guide for our present and future experimentation.

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[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27–30, 1963.

[2] Taken in part from dissertations submitted by Ammeraal, Kim, and Wilson to the Graduate School of Wayne State University in partial fulfillment for the degree of Doctor of Philosophy. Full experimental details not given here are in these dissertations.

[3] Supported by grants A–05384, National Institute of Arthritis and Metabolic Diseases, H-0439, National Heart Institute, and C-03345, National Cancer Institute, National Institutes of Health, Public Health Service; and by Predoctoral Fellowships from the National Institutes of Health and the National Science Foundation.

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[6] We wish to acknowledge the generous gift of hormones and synthetic peptides from Dr. J. D. Fisher, Dr. K. Hoffman, and Dr. C. H. Li.

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We will also report our current working hypothesis on the function of the hormone in this process of differentiation. Due to the complexity of the tissue, we have been unable to demonstrate directly (by time lapse cinemicrography) the differentiation of a nonpigmented melanoblast into a pigmented melanocyte. Therefore, our conception of this process is based, of necessity, on indirect evidence. However, we think it might be of some interest to present this hypothesis, for it correlates all the information available and represents our current approach to this problem.

Before presentation of the experimental results, we must define some of the terms used. A melanocyte is defined as a cell which is *capable* of synthesizing melanin. However, depending on the amount of melanin synthesized, a melanocyte may or may not appear visually to be pigmented. A melanoblast is defined as a cell which is *incapable* of synthesizing melanin but is potentially capable of giving rise to a melanocyte. The process of transformation, or differentiation, of a melanoblast into a melanocyte is called melanocytogenesis and the activity of the hormone(s) inducing this process is called melanocytogenic activity. In defense of the coined terms "melanocytogenesis" and "melanocytogenic activity," we can only say that these words are self-explanatory and that their usage is much more convenient than "the differentiation of melanoblasts into melanocytes" and "the activity to induce melanocyte formation from melanoblasts."

STATEMENT OF WORKING HYPOTHESIS

Our present hypothesis is as follows: 1) The melanoblasts in the xanthic goldfish are stem cells; 2) the formation of melanocytes from melanoblasts results from an asymmetric mitosis of the latter after stimulation by the proper hormone; 3) this hormonal activity depends on the same structure that is responsible for the classical MSH activity; 4) the effect of the hormone is exerted on, and perhaps required only by, melanoblasts prior to or during mitosis; 5) melanoblasts contain tyrosinase which is, however, inactive due to the simultaneous presence of a tyrosinase inhibitor; 6) the primary effect of the hormone is not at the genetic level, but at the cytoplasmic level and leads to the release or segregation of tyrosinase from its inhibitor during mitosis.

Some experimental results forming the basis of this hypothesis will be discussed in the following sections. The details of the experimental conditions and results either have been or will be reported elsewhere (*2–6, 8–11*).

CORRELATION OF STRUCTURE WITH MELANOCYTOGENIC ACTIVITY

Through the generous gifts of Professor K. Hoffman and Professor C. H. Li, we have been able to determine the melanocytogenic activity

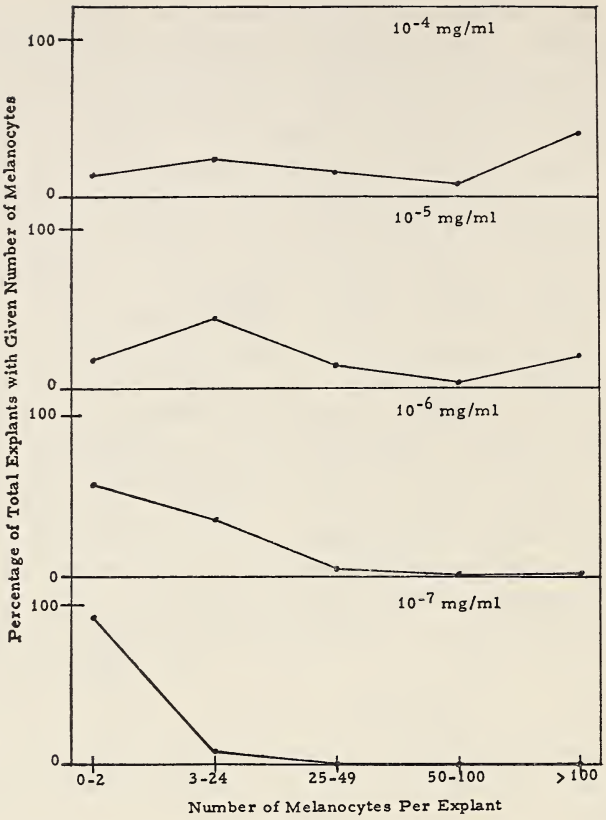
of pure α- and β-MSH, α-ACTH, alkali-treated α-ACTH, and several synthetic peptides. The details of experimental conditions and results will be published elsewhere, but the summary and one example, the comparison of the activity of ACTH and α-MSH, will be given here. Briefly, the assay is based on the ability of different concentrations of the peptides (or hormones) to cause the appearance of melanocytes in explants of standard size, treated with peptides under standard conditions. With each concentration of a peptide, the explants used are divided into groups containing 0 to 2, 3 to 25, 26 to 50, 51 to 100, and >100 melanocytes. With no hormone treatment, all the explants should fall into the first group (0–2 melanocytes per explant). With optimal hormone treatment, usually 80 percent or more of the explants fall into the last category (>100 melanocytes per explant). For the comparison of the melanocytogenic activity of different peptides, the distribution of explants into these groups is plotted graphically for all concentrations of hormone tested. The concentrations of the peptides required to give an "intermediary" response are estimated and compared. Such plots for α-MSH and ACTH are shown in text-figures 1 and 2. From these data, it was estimated that α-MSH is slightly more than 30 times as active as α-ACTH. The melanocytogenic activities of other hormones and peptides were compared to the activity of α-MSH in similar manner. A summary of the results of such experiments is shown in table 1 [from (*11*)]. In this table, the relative melanocytogenic activities of the different peptides are listed side by side with their relative activity in the classical MSH assay (melanin granule dispersion). It can be seen that, although minor differences exist, there is, on the whole, excellent correspondence in these two activities.

INVOLVEMENT OF MITOSIS IN THE HORMONE-INDUCED MELANOCYTOGENESIS

The involvement of mitosis in the hormone-induced melanocytogenesis is deduced from experiments with 3 well-known mitotic inhibitors: colchicine (*5*), urethan, and mercaptoethanol.[7] These compounds are known to inhibit mitosis at concentrations of 0.3 μg per ml (*6*), 7.5 mg per ml (*12*), and 5×10^{-3} M (*13*), respectively. In our experiments, it was found that these are the minimal concentrations of these agents required to inhibit completely the melanocytogenic effect of hormone added simultaneously with the inhibitor. The complete inhibition of melanocytogenesis by all 3 mitotic inhibitors and the correspondence of the minimal concentrations of these agents necessary to inhibit melanocytogenesis and mitosis are consistent with mitosis being obligatory in hormone-induced melanocytogenesis.

This conclusion is also supported by another type of experiment with 2 inhibitors, colchicine and mercaptoethanol. In these experiments, the

[7] The work with urethan and mercaptoethanol is still unpublished but is fully described in (*10*).



TEXT-FIGURE 1.—Response to α-MSH at the concentrations shown. If 10^{-3} mg per ml of α-MSH was used, over 80 percent of the explants fell into the group with more than 100 melanocytes per explant.

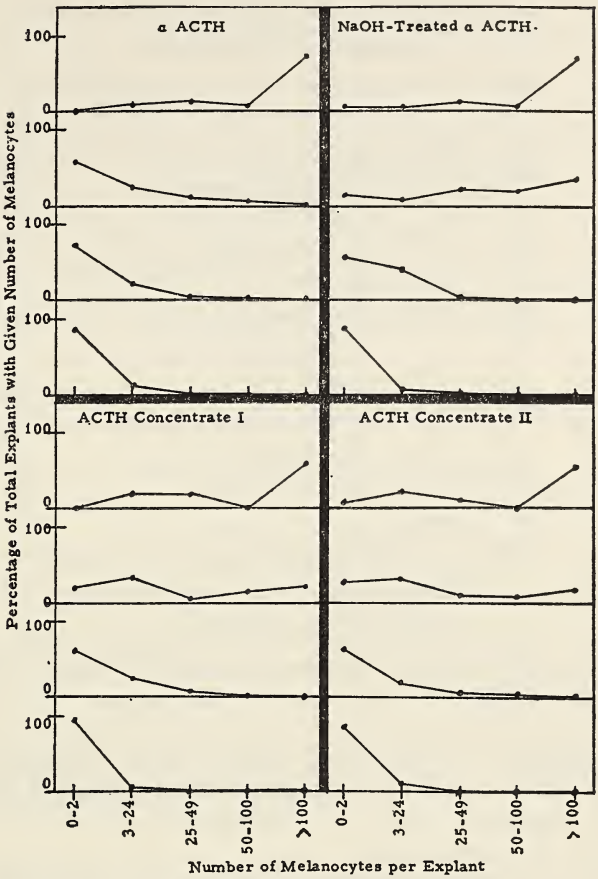
TABLE 1.—Comparison of melanocytogenic activity to classical MSH activity of various peptides related to MSH

Peptide	Melanocyte formation activity*	Melanin dispersion activity†
α-MSH	1	1
β-MSH	Slightly $>10^{-1}$	1.2×10^{-1}
Armour ACTH concentrate	$\sim 3 \times 10^{-2}$	10^{-2}‡
α-ACTH	Slightly $<3 \times 10^{-2}$	6×10^{-3}
NaOH-treated α-ACTH	Slightly $>3 \times 10^{-2}$	1.1×10^{-2}
Glu-his-phe-arg-try-gly	$<10^{-5}$	2×10^{-5}
Ser-10-OH	$\sim 10^{-3}$	2.9×10^{-4}
Ser-13-NH_2	Slightly $<10^{-1}$	1.9×10^{-1}
Ser-16-OH	Slightly $>10^{-2}$	3.7×10^{-2}
Ser-17-OH	10^{-1}–10^{-2}	Almost the same as α-ACTH
Ser-20-NH_2	$\sim 10^{-2}$	1.1×10^{-2}

*Expressed as the specific activities of the peptides, assuming the specific activity of α-MSH to be 1.

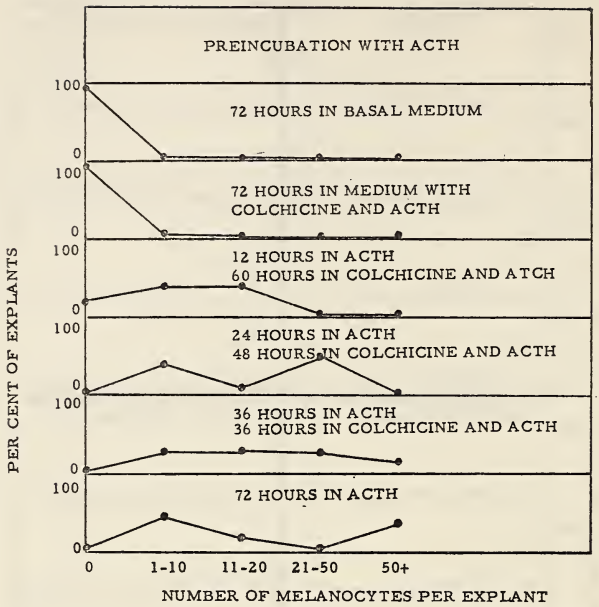
†Expressed as specific activities with that of α-MSH defined as 1. Data are collected from reports of other workers. References may be found in (*11*), from which this table was taken.

‡The melanocytogenic activity and the classical MSH activity of the Armour ACTH concentrate were obtained with 2 different lots with the same ACTH activity per mg.



TEXT-FIGURE 2.—Response to 3 samples of ACTH. The concentrations used were, from *top* to *bottom* in each series, 3×10^{-2}, 3×10^{-4}, 3×10^{-5}, and 3×10^{-6} mg per ml. The response to 3×10^{-4} mg of α-ACTH per ml was between the responses to 10^{-5} mg and 10^{-6} mg of α-MSH per ml. Similarly, the response to 3×10^{-5} mg of α-ACTH per ml was between the responses to 10^{-6} mg and 10^{-7} mg of α-MSH per ml. Alkali treatment of ACTH led to slight increase in activity. The figures with ACTH concentrate I and II are, respectively, plots of the composite accumulated data in all experiments and with only the data obtained with the same fish used for ACTH assay. The 2 plots are in good agreement with each other, which indicates the reliability of the assay procedure.

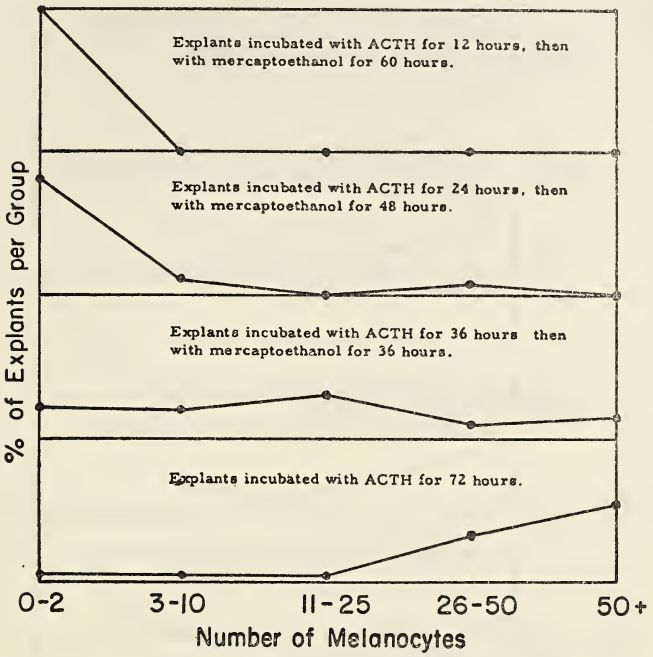
mitotic inhibitor was not added with the hormone, but several hours after the hormone. If the explants were first incubated with hormone for 12 to 36 hours, the subsequent addition of the mitotic inhibitor failed to give complete inhibition of melanocytogenesis (text-figs. 3 and 4). Furthermore, if the explants were fixed after only 12 to 24 hours of incubation with hormone, no melanin granules were visible. These results point to the fact that the process of hormone-induced melanocytogenesis and melanin synthesis can be separated into at least 2 phases. In the first phase, the melanoblast undergoes a process inhibited by the



TEXT-FIGURE 3.—Gradual emergence of colchicine resistance as a result of incubation with hormone. The number of explants in the curves (from *top* to *bottom*) are: 27, 10, 10, 10, 7, and 11.

miototic inhibitors. In the second phase, there is melanin synthesis not inhibited by the mitotic inhibitors. Such behavior of the melanoblasts are in agreement with our working hypothesis that mitosis is obligatory in melanocytogenesis.

A third line of evidence in support of this hypothesis was obtained with the so-called synchronized melanoblasts. In an attempt to synchronize the melanoblasts, explants were incubated first in basal tissue culture medium containing 1 μg per ml of colchicine. After 2 to 3 days, the coverslip with the explants was removed, rinsed with basal tissue culture medium, and reincubated with medium containing the hormone. After a few hours of incubation with hormone, the explants were subjected to a second treatment by the mitotic inhibitors. If the second dose of mitotic inhibitor was added within the first 8 hours after the removal of the explants from colchicine preincubation, melanocytogenesis was completely or nearly completely inhibited. If the second dose of inhibitor was added 15 or more hours after the preincubation, there was either no inhibition (with colchicine) or only partial inhibition (with urethan and mercaptoethanol) (text-figs. 5, 6, and 7). With each individual fish, this transition from complete sensitivity to complete (in the case of colchicine) or partial resistance to the mitotic inhibitor occurred usually within 3 hours. Such a sudden transition suggested the synchronization of the melanoblasts by the preincubation with colchicine. Furthermore, the transition time was approximately the same with all 3 mitotic inhibitors, in agreement



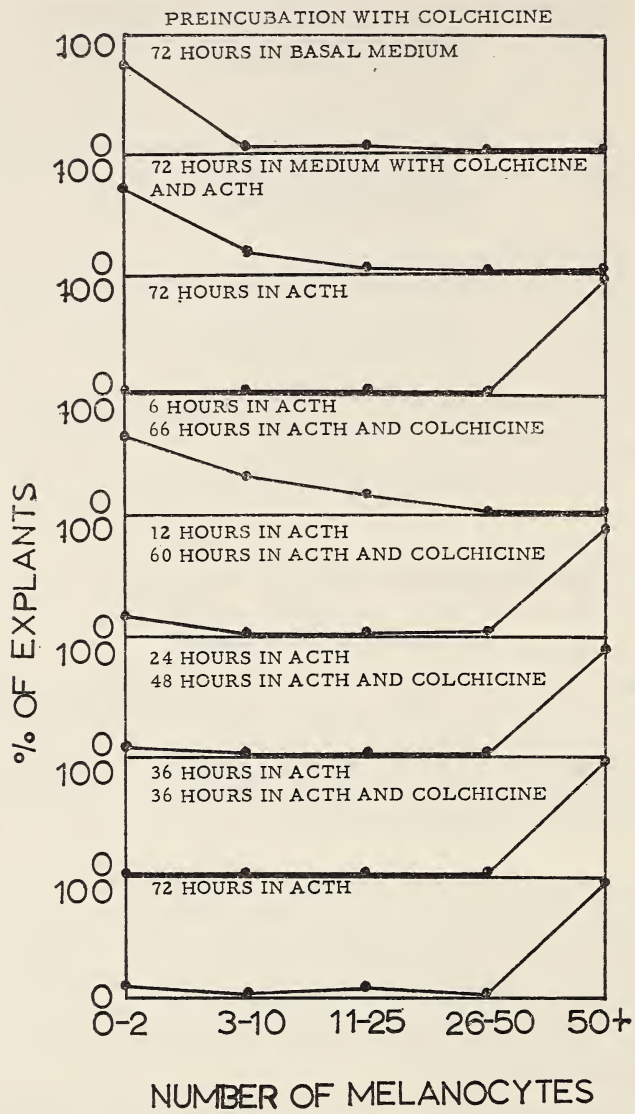
TEXT-FIGURE 4.—Gradual emergence of mercaptoethanol resistance as a result of incubation with hormone. By comparison with text-figure 3, under the same conditions the response obtained in the presence of mercaptoethanol was always inferior to that obtained in the presence of colchicine. This has been discussed in detail in (*10*) and will not be treated here. The number of explants in the curves (from *top* to *bottom*) are: 108, 16, 21, and 19.

with the hypothesis that the inhibitory effect of these 3 compounds is exerted on the same process, namely, mitosis. However, the success of the synchronization experiments varied from one batch of fish to another. In the experiments reported here, the degree of synchronization was very good. In other groups of fish, the degree of synchronization was much poorer, with only partial synchronization even after 72 hours of preincubation with colchicine (*11*).

In summary, the results of the studies with these 3 classical mitotic inhibitors are in complete agreement with the obligatory involvement of mitosis during melanocytogenesis, and, from our prejudiced point of view, readily explicable only by such a hypothesis and thus furnish strong support for the hypothesis.

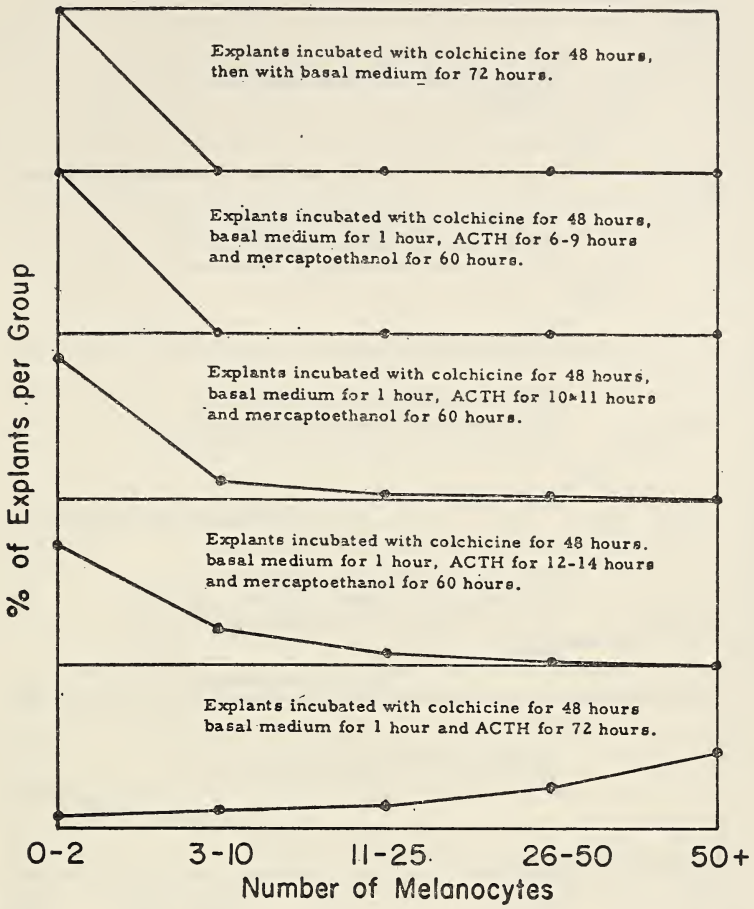
MELANOBLASTS AS STEM CELLS?

It is our current belief that the melanoblasts in the xanthic goldfish are stem cells. The reasons for this, and the weaknesses of the reasoning, are briefly listed: 1) There is no indication that melanocytes appear in



TEXT-FIGURE 5.—Response of "synchronized" melanoblasts to hormone and colchicine. If the preincubation was carried out in the absence of colchicine, a substantial number of colchicine-resistant cells would be present 4 hours after hormone treatment. With longer duration of hormone treatment, there was a gradual increase of colchicine-resistant melanocytes. This is illustrated and discussed in (*11*). The number of explants in the curves (from *top* to *bottom*) are: 14, 14, 17, 15, 14, 9, 9, and 13.

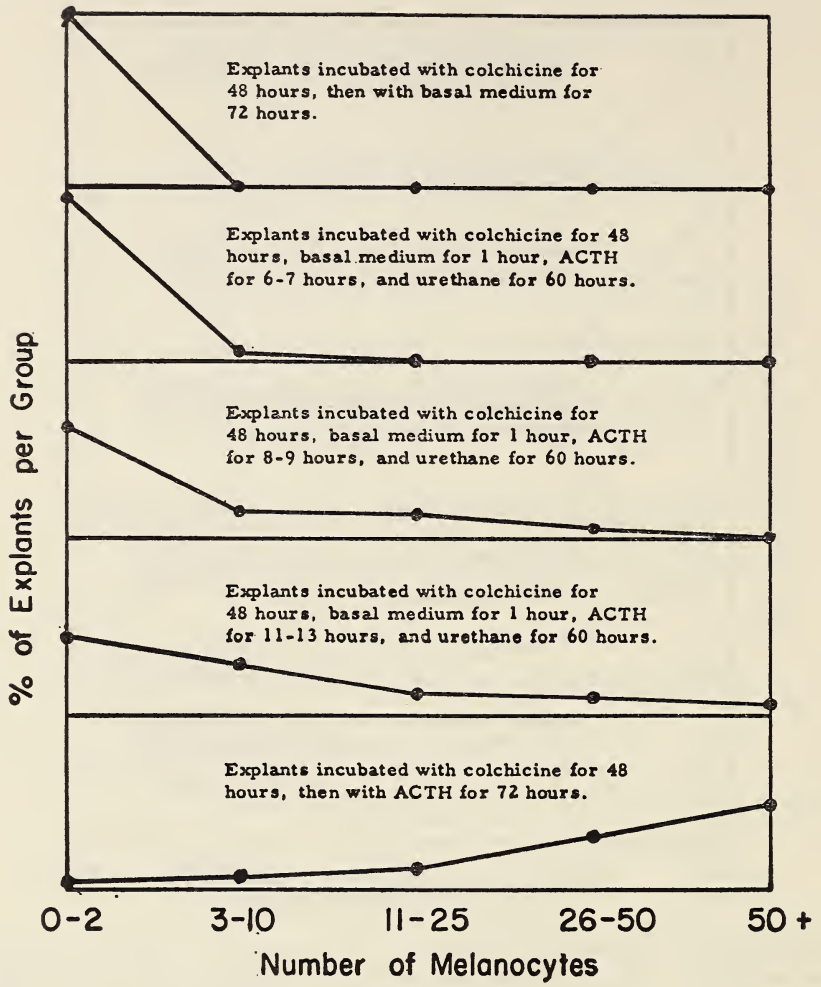
pairs as might be expected if the 2 daughter cells resulting from the mitosis of a melanoblast are identical. The defect in this argument is that melanocytes are known to migrate and that, even if they are paired immediately after mitosis, they may have traveled far apart by the time



TEXT-FIGURE 6.—Response of "synchronized" melanoblasts to hormone and mercaptoethanol. Again, in comparison to text-figure 5, the response was always poorer in the presence of mercaptoethanol than in the presence of colchicine. The number of explants in the curves (from *top* to *bottom*) are: 81, 55, 66, 91, and 79.

of fixing and microscopic examination. 2) Carlson and his co-workers have shown in grasshoppers that the neuroblasts, closely related to the melanoblasts in their embryonic origin, are stem cells (*14*). The weakness of the argument is that these neuroblasts may well be a very special case instead of a typical example of the fate and behavior of all or most cells of neural crest origin. 3) If a fish is subjected to continuous "stress," (by X-ray irradiation) it will remain in the pigmented state permanently (*15*). Since the melanocytes in this variety of goldfish have never been observed to divide and have a lifespan of the order of 10 days or less,[8] one is led to assume a continuous production of melanocytes. Such a continuous formation of melanocytes can be readily explained by the assump-

[8] This is evidenced by the complete loss of pigmentation of stressed xanthic goldfish within 10 days after initiation of stress, as illustrated in (*3*).



TEXT-FIGURE 7.—Response of "synchronized" melanoblasts to hormone and urethan. The number of explants in the curves (from *top* to *bottom*) are: 59, 48, 32, 60, and 42.

tion that the melanoblasts are stem cells. The weakness of this argument is that, although the lifespan of melanocytes is known with NaCl-stressed fish, it is not known with permanently stressed fish.

Such is the status of the arguments for the hypothesis that in the common goldfish the melanoblasts are stem cells. It is clear that a definitive statement cannot be made at the present time, but must await further experimentation.

FUNCTION OF THE HORMONE

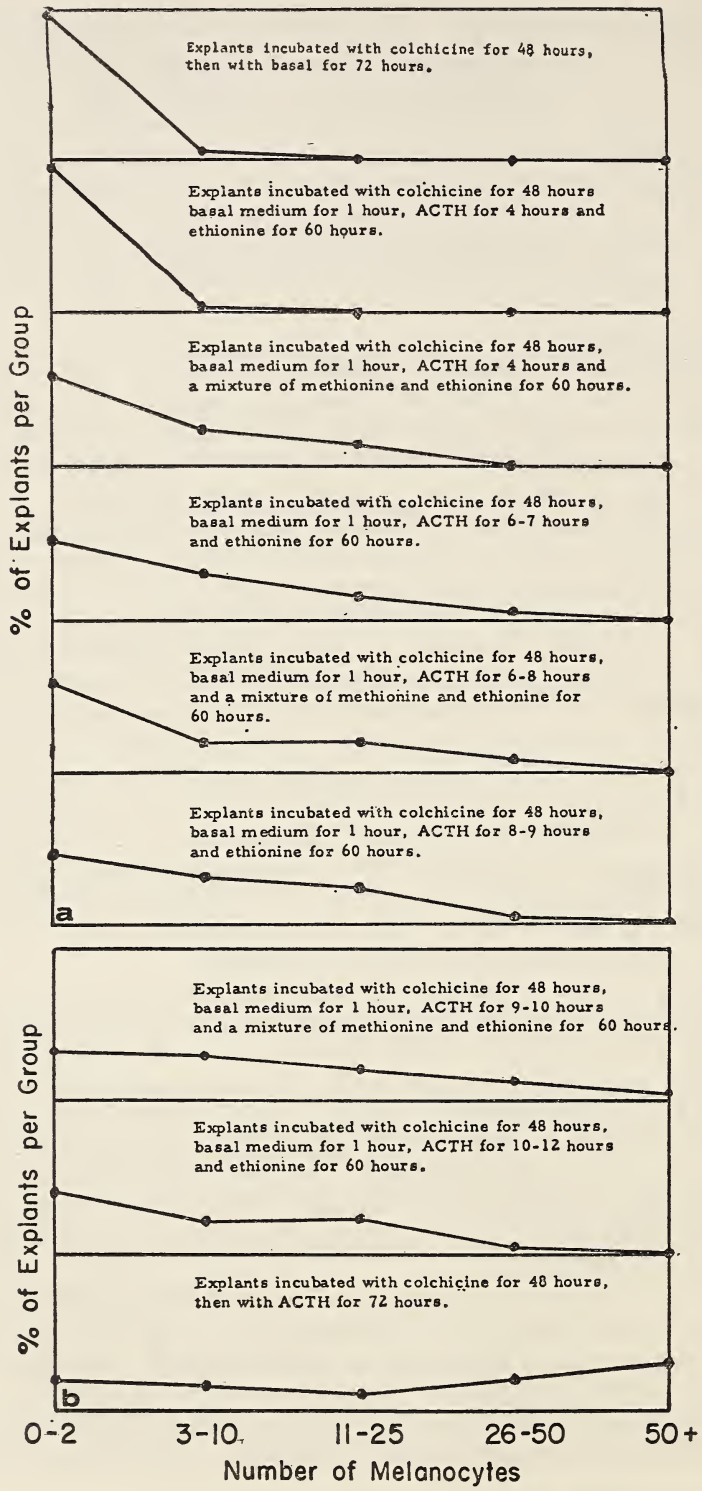
The function of the hormone in melanocytogenesis is at the present a matter of conjecture. Indeed, one does not even know what are the

target cells of the hormone. All attempts to induce melanocytogenesis in monolayer cells, whether obtained by trypsinization or by direct "outgrowth" from the explants, have thus far been unsuccessful. It is, therefore, not possible to say whether the hormone acts directly on the melanoblasts, or whether the hormone acts on some other cells which in turn act on the melanoblasts. In either case, however, these melanoblasts should be acted on by some outside agent and one could meaningfully ask, without identification of this agent, what is its mode of action. For the purpose of simplicity, we shall assume in the discussion that this outside agent is the hormone, or, in other words, that the hormone acts directly on the melanoblasts.

The one obvious qualitative difference between the melanoblast and the melanocytes is the absence or presence of melanin, the synthesis of which required tyrosine, oxygen, and a copper-enzyme tyrosinase. Since tyrosine and oxygen must be present in all cells, the difference between the two types of cells must be due to the enzyme tyrosinase. The level of this enzyme seems, therefore, the obvious place to look for the action of the hormone. In previous communications, the tyrosinase content of the skin of completely xanthic, partially black, or totally black goldfish has been reported (*3*, *8*). The conclusions from these studies are pertinent to the present discussion: 1) When xanthic goldfish are stressed, there is a several fold increase in tyrosinase which precedes maximum pigmentation. 2) A considerable amount of tyrosinase is present in the homogenate of the skin of completely xanthic goldfish, even though no melanocyte was visible by microscopic examination. 3) There is present in the soluble portion of the skin homogenate a tyrosinase inhibitor which is either a protein or protein-bound. The presence of tyrosinase in the skin homogenate of completely xanthic fish indicated that the melanocytogenic effect of the hormone cannot be explained by a simple "derepression" of the tyrosinase gene. This conclusion has now received further experimental support which is described.

During a survey of the effects of various amino acid, purine, and pyrimidine analogues on melanocytogenesis (*10*), it was observed that these compounds can completely inhibit melanocytogenesis, although the concentrations required for complete inhibition are unusually high, due probably to the low permeability through the skin. Among the compounds tested was ethionine which, at 10 mg per ml, could completely inhibit hormone-induced melanocytogenesis. Although this concentration is extremely high, the inhibition is reversed by the further addition of 1 mg per ml of methionine, which indicates that ethionine is acting as an analogue of methionine.

When a synchronized population of melanoblasts (synchronized by colchicine preincubation as described earlier) were incubated with hormone for varying periods and then treated with ethionine, a transition from complete inhibition to partial inhibition was observed at about 7 hours after the removal of colchicine (text-fig. 8). The most significant point here is that the resistance to ethionine seems to appear earlier than the


Explants incubated with colchicine for 48 hours, then with basal for 72 hours.
Explants incubated with colchicine for 48 hours basal medium for 1 hour, ACTH for 4 hours and ethionine for 60 hours.
Explants incubated with colchicine for 48 hours, basal medium for 1 hour, ACTH for 4 hours and a mixture of methionine and ethionine for 60 hours.
Explants incubated with colchicine for 48 hours, basal medium for 1 hour, ACTH for 6-7 hours and ethionine for 60 hours.
Explants incubated with colchicine for 48 hours, basal medium for 1 hour, ACTH for 6-8 hours and a mixture of methionine and ethionine for 60 hours.
Explants incubated with colchicine for 48 hours, basal medium for 1 hour, ACTH for 8-9 hours and ethionine for 60 hours.
a
% of Explants per Group
Explants incubated with colchicine for 48 hours, basal medium for 1 hour, ACTH for 9-10 hours and a mixture of methionine and ethionine for 60 hours.
Explants incubated with colchicine for 48 hours, basal medium for 1 hour, ACTH for 10-12 hours and ethionine for 60 hours.
Explants incubated with colchicine for 48 hours, then with ACTH for 72 hours.
b
% of Explants per Group
0-2
3-10
11-25
26-50
50+
Number of Melanocytes

resistance to the mitotic inhibitors colchicine, mercaptoethanol, and urethan. One may thereby conclude that protein synthesis after mitosis is not obligatory for the synthesis of melanin. This is in agreement with the conclusion arrived at earlier from tyrosinase studies and argues against the derepression of the tyrosinase gene as the primary function of the hormone. Rather, we may postulate that the melanocytogenic effect of the hormone is achieved by the removal of a tyrosinase inhibitor. Since the phenotypic appearance of active tyrosinase does not occur in melanoblasts which have been treated with hormone but have not undergone mitosis, one can postulate further that hormone does not cause the destruction of the inhibitor in melanoblasts, but rather causes a segregation of tyrosinase and its inhibitor during mitosis. The end result of such action is that one of the daughter cells now possesses active tyrosinase and can proceed to synthesize melanin and become a visibly recognizable melanocyte.

SUMMARY

The melanocytogenic activities (ability to stimulate the differentiation of unpigmented melanoblasts into pigmented melanocytes) of α-MSH, β-MSH, ACTH, and several synthetic polypeptides were determined. The activity of these compounds, relative to α-MSH, paralleled their classical MSH activity (dispersion of melanin granules), with α-MSH the most active.

Based on indirect evidence, accumulated with the use of inhibitors and from enzyme studies, a hypothesis to describe this process of hormone-induced melanocytogenesis was proposed. The principal features of this hypothesis are: 1) Melanoblasts in the xanthic goldfish are stem cells, and 2) the primary function of the hormone is not to derepress the tyrosinase gene but to cause the segregation of tyrosinase from tyrosinase inhibitor during mitosis.

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Text-figures 8*a* and *b*.—Response of "synchronized" melanoblasts to hormone and ethionine. Even after 7 to 8 hours of hormone treatment, a considerable number of ethionine-resistant melancytes are already present. *Note*, in the second and third curve from the *top*, the reversal of ethionine inhibition by methionine. The number of explants in the curves are (from *top* to *bottom*): 8a to 8b: 60, 57, 40, 39, 36, 37, 77, 33, 73, and 59.

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HORMONE-SECRETING CULTURES OF ENDOCRINE TUMOR ORIGIN [1, 2]

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The dispersed cell culture systems described here have been developed with the aim of investigating the factors involved in the maintenance of differentiated properties in mammalian cells (*1*). These systems are appropriate for such studies because specialized functions seem to depend for their maintenance on conditions found in the whole organism and do not possess a great degree of stability under certain "*in vitro*" conditions. When mammalian cells are removed from the animal and placed in dispersed cell culture, they tend to lose the physiologically specialized functions which characterized them in the integrated status (*2*).

The pertinent question is whether the specialized properties are irretrievably lost in culture, *e.g.*, by loss of the specific cell type by selection or the loss of necessary chromosomal material, or whether cells in culture retain the potential for expressing these properties under suitable conditions. If the retention of potential can be demonstrated as generally present in cultures in which the overt expression of differentiated properties has disappeared, it becomes experimentally feasible to use these cultures for study of the factors controlling the expression of differentiated properties.

ALTERNATE ANIMAL AND CULTURE PASSAGE

If cells lose their specific enzymatic activities because of the inadequacy of the culture environment, then only the reconstitution of an environment with the proper composition for a sufficiently protracted period would allow one to ascertain the stability of the functional change induced in these cells by the passage through culture. It is apparent how complex this problem can be if each different cell type requires a different, specially supplemented, nutrient medium for the expression of its specialized func-

[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27–30, 1963.

[2] Aided in part by grant C-04123 from the National Cancer Institute, National Institutes of Health, Public Health Service, and grant G-19457 from the National Science Foundation.

[3] Postdoctoral Trainee 2G-212, Institute of General Medical Sciences, National Institutes of Health.

[4] We thank Miss Jean Thivierge and Mrs. Maria Brasats for their competent technical assistance. The following graduate students have contributed to the progress of this work through their participation in the Biochemistry Techniques course: Mr. Robert Pierson, Mr. Kenshi Satoh, Mr. Marvin Goldlust, and Dr. Lucien Cuprak.

tion. A possible means of overcoming this difficulty, particularly in the preliminary phase of an investigation of this kind, is the use of functional tumors. Since these cells are not subject to growth control in the animal and are transplantable, they can be replaced in their usual environment at any stage of culture simply by grafting them back to the animal: The function of the new tumor arising from this culture can then be tested. Also, the effect of replacing a particular cell population in the proper environment can be followed for a protracted period through serial animal passages; the advantage of this procedure will be illustrated later. Also, a given cell line that has been established through culture, sometimes by a particular treatment, can be obtained in large quantity even if growth *in vitro* should initially be insufficient. This procedure of alternately passaging cells through culture and animal may also be generally useful for obtaining cells more suitable for culture experiments. When these tumors are placed in culture for the first time, growth in the initial cultures is often poor and the cells usually survive for only a few weeks. When the cells surviving this initial culture period are reinjected into mice, new tumors (culture-derived tumors) are formed, which differ from the original line in that their growth in culture is greatly improved (fig. 1). One possible explanation for this improved growth is that during the initial culture period a selection occurs for pre-existing types of cells that can better survive the conditions of culture. An alternative explanation is that conditions in culture induce a stable modification of the metabolism of these cells, enabling them to grow better in culture. This treatment would only favor the cancerous endocrine cells, since cells from the supporting tissue that survive the culture period would not contribute much to the formation of the new tumor. Cultures of culture-derived tumors produce hormones at a greater rate and for a longer time period than do those derived from the original tumor line. These variations in endocrine behavior will be considered further in a later section.

For these reasons, the alternate passage through culture and animal was routinely used in the present phase of this investigation. The functional transplantable mouse tumors of pituitary and adrenal origin selected for this work were developed and extensively characterized by Dr. J. Furth and associates (3–8).

MEASUREMENT OF SPECIFIC FUNCTION

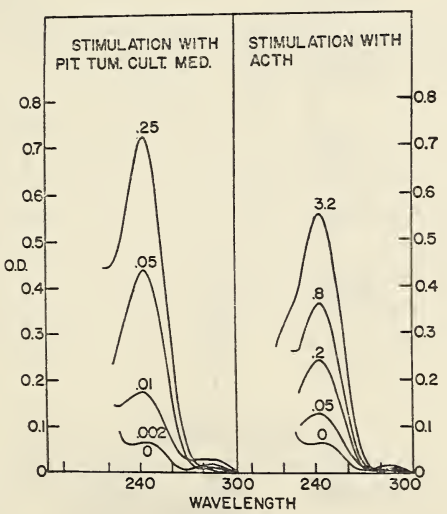
The end products of the specific pathway of the adrenocortical tumor cells have in common the Δ^4-3-keto structure in ring A, which is responsible for the characteristic ultraviolet absorption spectrum of these compounds with a maximum at 242 mμ. Stimulation with corticotropin greatly increases the production of these compounds by the adrenocortical tumor cells, and the specific function of these cultures is therefore measured as total Δ^4-3-ketosteriod output under corticotropin stimulation. The method of measuring the steroids secreted by these cultures

is a variation of the one developed by Saffran, Grad, and Bayliss (*9*). The medium is aspirated from the adrenal tumor cultures and replaced with an incubation buffer supplemented with ACTH. After a suitable incubation period, usually 2 hours, the buffer is harvested and extracted with methylene chloride which is then removed by evaporation, and the residue is dissolved in ethyl alcohol. The ultraviolet absorption spectra of these alcoholic solutions are then measured spectrophotometrically. The ultraviolet absorption spectra of the steroid material produced by adrenal cultures under stimulation by graded doses of corticotropin are shown in text-figure 1.

The pituitary tumor secretes only the trophic hormone for the adrenal cortex. From what has been said in the preceding paragraph, it follows that the adrenal tumor cultures can be used for the quantitation of the specific function of the pituitary cultures. Text-figure 1 shows the ultraviolet spectra obtained when adrenal tumor cultures were stimulated with graded doses of pituitary tumor culture medium. The assay is specific and highly sensitive, and because of the simplicity of the method, the specific function of corticotropin-producing cells can be quantitated reliably and routinely.

REVERSIBILITY OF PHENOTYPIC ALTERATIONS

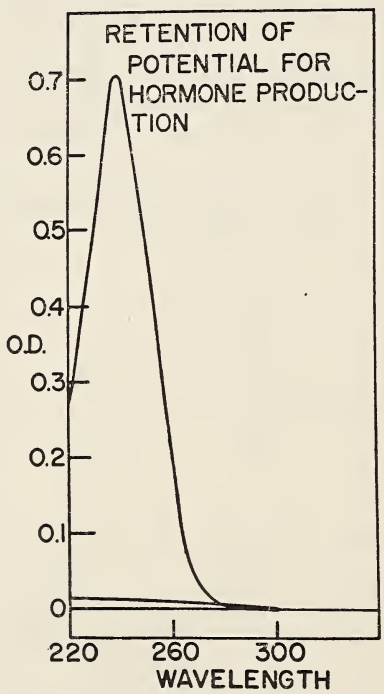
As previously mentioned, it is of prime interest to determine whether cells that have lost function in culture retain the ability to express this activity under favorable conditions. We have found in our experi-



TEXT-FIGURE 1.—Curves on *right* represent the absorption spectra obtained when adrenal tumor cultures were stimulated with 0, 0.05, 0.2, 0.8, and 3.2 milliunits of Armour's ACTH per ml incubation medium. Curves on *left* were obtained when the cultures were stimulated with 0, 0.002, 0.01, 0.05, and 0.25 ml of pituitary tumor culture medium per ml incubation medium.

ments that, if cultures in which the specific function had disappeared were injected into mice, the resulting tumor was composed of cells with fully restored function. Text-figure 2 shows an absorption curve of an adrenal tumor, cultured for several months, in which stimulation of the specific pathway by the pituitary hormone did not result in the usual increase in the rate of production of Δ^4-3-keto compounds (bottom curve). It such a culture was then injected into mice, a new tumor was obtained whose cells when cultured were now fully responsive to the regulatory hormone (top curve). Essentially the same results were obtained with corticotropin-secreting cells.

Other similar experiments have revealed that the alterations in the physiology of these cells observed in culture are, to varying degrees, stable. At times the alterations appear permanent but they are still reversible. When cells lose their ability to secrete hormone in culture, this new, nonfunctional state is stable indefinitely if the cells are continuously propagated in culture but, if they are again propagated as a tumor, the function may be restored by a single animal passage. Sometimes, several passages through the animal host may be required before specific function is regained: No specific enzymatic activities were detectable in adrenal tumor cells kept in culture for 200 days and which



Text-figure 2.—Adrenal tumor cultured for several months and stimulated with ACTH (*bottom* absorption curve). These nonfunctional cultures were injected into mice and the new tumors that grew were plated and the cultures stimulated with ACTH. *Top* absorption curve was obtained. Both tests were performed under the same conditions.

had multiplied by a factor of 10^{10}. The resulting tumor was propagated by animal passage for a year, during which time no hormonal activity was detectable either in the animal or in culture. After an additional 6 months of passage, this tumor line progressively regained its hormonal activity, but the course of restoration of the activity was peculiar. At the beginning, stimulation with corticotropin resulted in the formation of some compounds, possible precursors, which did not show the characteristic absorption peak at 242 mμ but could be further transformed by incubation with the standard culture line to Δ^4-3-ketosteroids.

In the first few passages after its initiation, the adrenal tumor line lost its ability to synthesize steroids with the α-ketol side chain. This activity was never observed in subsequent tests during the next 8 years of animal passage. After 2 years of alternate animal-to-culture passage, pooled extracts from various adrenal tumor cultures contain 10 percent of the secreted steroids with the α-ketol side chain. Thus, an activity lost in the animal was restored by culture passage.

Also, the cells of the corticotropin-secreting cultures can increase by a factor of about 10^{50} after 2 years in culture and still regain hormonal activity after animal transfers.

Another change occurring in culture is the increase of growth rate. After several months, these cultures usually shift from a generation time of 5 days to a generation time of 2 days. When these cells in culture acquire a rapid rate of growth, the tumors arising from them grow rapidly and must be transferred every 7 days, while the original tumor line grows so slowly as to require transfer only every 60 days under the same conditions of inoculation.

Passaging these tumor cells through culture has also resulted in tumor lines that differ from one another in the rate and stability of hormone secretion in culture and the rate of growth. One of these variant lines will be discussed in some detail later.

These observations support the view that the cells undergo alterations in their physiology during passage through culture and animal. It seems unlikely that the changes observed in these populations could be due to selective growth of pre-existing cell types because of the large factor (10^{50}) by which nonfunctional cultures can multiply and still give rise to functional tumors. Also, some of the activities necessary for over-all steroidogenesis can still remain high while others have almost completely disappeared.

Ascorbic Acid

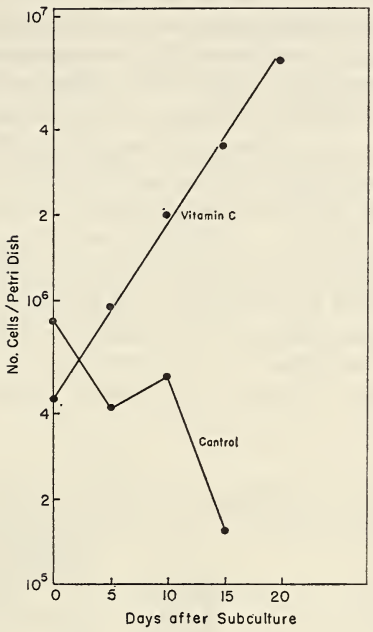
Currently it is believed that adrenocorticotropin (ACTH) acts by raising the level of 3′,5′-cyclic adenosine monophosphate (AMP) in the adrenal cortex. The elevated level of cyclic AMP activates phosphorylase which in turn breaks down glycogen, yielding glucose-6-phosphate for the generation of triphosphopyridine nucleotidase (TPNH). TPNH is a required cofactor in several of the hydroxylations involved in steroidogenesis, and its availability is postulated to be rate-limiting in the

reaction whereby the side chain of cholesterol is cleaved, yielding pregnenolone and isocaproic aldehyde. This hypothesis of steroidogenesis in the adrenal cortex has been derived from experiments with normal adrenocortical tissue (*10*). To determine whether the behavior of the adrenal tumor cultures is similar to that of normal adrenocortical systems, the activity of those agents known to mediate corticotropin action was tested on cultures (*11*). These agents have been found to play the same role as in the normal gland: The over-all rate of steroid synthesis is stimulated by 3′,5′-cyclic AMP, and reduced TPN; pregnenolone and its Δ^4 derivative are rapidly converted to Δ^4-3-ketosteroids in the absence of corticotropin. If the intracellular content of the substrate (cholesterol) utilized by the early enzymes of the specific pathway is low, corticoidogenesis proceeds at a very low rate in the presence of ACTH. In these respects, adrenal tumor cultures are similar to the normal adrenal cortex.

Another characteristic of the adrenal cortex gland is its high content of ascorbic acid which drops rapidly after ACTH administration. Neither the significance of this change, which seems to precede corticoidogenesis, nor the importance of the high intracellular content of vitamin C is, at present, completely clear. When cultures of adrenal tumors were examined, although tumor tissue contained high levels of ascorbic acid (12 μg/mg protein), none was detectable in cultures kept in medium not supplemented with the vitamin (*11*). Nevertheless, the steroidogenesis by these cultures was not adversely affected. They responded for months to ACTH stimulation with an increase in the rate of steroidogenesis 3 to 4 times higher than that of the tumor tissue slices. Exposure of these cultures to media enriched with graded concentrations of ascorbic acid revealed the presence of a mechanism which maintains the intracellular concentration of the vitamin at a much higher level than the extracellular one. ACTH slows the rate of uptake of ascorbic acid and can partially deplete ascorbic acid taken up previously by the cells (*11*). In attempting to maintain the physiological intracellular concentration of ascorbic acid in culture, we encountered serious technical difficulties because of the rapid oxidation of this molecule. Our experience emphasizes the obstacles in maintaining a proper *in vitro* environment. Apparently, adrenocortical cells in the body concentrate ascorbic acid, which is synthesized in the liver and supplied to the circulation at a steady rate. To approximate this condition *in vitro*, adrenal tumor cultures were supplemented with ascorbic acid daily to a final concentration of 0.002 g per 100 ml. This treatment, as seen in text-figure 3, results in an enhanced growth which is particularly evident after subculture.

Separate Decline in Culture of Individual, Specialized Adrenocortical Properties

To elucidate the mechanism by which adrenal tumor cells lose their specific function in culture, it is necessary to first acquire a more detailed knowledge of the progression of events leading to this loss. One



TEXT-FIGURE 3.—Adrenal tumor cultures were supplemented daily with ascorbic acid to a final concentration of 0.002 g per 100 ml. After 60 days of culture, the cells were subcultured and the ascorbic acid regimen maintained. *Upper* growth curve was obtained after subculture. Control cultures, treated identically except for the ascorbic acid supplementation, yielded *lower* growth curve.

approach is to follow separately in culture each of the individual activities that make up the complex of adrenal specific properties. Thus, it should be possible to determine whether the over-all loss of steroid secretory activity is due to a limited lesion of one or a few of the necessary enzymes or to a more generalized depression of the many enzymatic steps involved in steroidogenesis. This approach may also reveal the primary events leading to the loss of this function. Preliminary results are of interest.

The properties we have chosen are: steroid secretion upon ACTH stimulation, 3-β-ol steroid dehydrogenase activity, conversion of pregnenolone to Δ^4-3-ketosteroids, cholesterol content, and ability to concentrate ascorbic acid. To follow the decline of these properties in culture, we have selected a variant tumor line whose hormonal activity is never very high but declines rather rapidly in culture. This line was obtained by

alternate passage through culture and animal and is designated "the low function, unstable line." Table 1 presents the values for these various activities in cultures of this line after different times in culture, of the standard line and of control HeLa cell cultures. It is evident that all these activities disappear eventually, and more or less simultaneously. However, 3-β-ol steroid dehydrogenase activity remained relatively high after the other activities were gone. Also, the ability to concentrate ascorbic acid and the high cholesterol content, characteristic of adrenocortical tissue, tended to be absent from the beginning of the culture period. Some cultures that are nonfunctional by the criterion of their ability to secrete steroids under ACTH stimulation can secrete steroids when furnished exogenous cholesterol. Possibly, the primary effect of culturing these cells is that the mechanism for concentrating ascorbic acid and accumulating cholesterol is disordered, and the loss of the other specific properties results. The present observations do not reveal whether the enzymes responsible for these various specialized activities are actually absent or are inhibited. Future experiments must be directed toward these questions.

TABLE 1.—Decline of individual properties

Test	Standard adrenal tumor culture	Unstable, low function adrenal tumor culture (day)			HeLa culture
		4	20	30	
Δ^4-3-Ketosteroids formed per mg protein per hour under maximal ACTH stimulation	4. 00	0. 78	0. 11 0. 83*	0. 18	<0. 01
Δ^4-3-Ketosteroids formed per mg protein per hour in presence of 70 μg per ml pregnenolone	9. 34	7. 20	4. 60	1. 90	<0. 01
Δ^4-3-Ketosteroids formed per mg protein per hour in presence of 70 μg per ml Δ^4-pregnene-20α,3β-diol	16. 50	12. 30	7. 50	5. 20	<0. 01
Cholesterol content μg per mg protein	65. 50	26. 30	22. 70	19. 00	15. 50
Ascorbic acid concentrated by cells μg per mg protein after 2 hours in 0.01 g per 100 ml ascorbate	12. 00	2. 50	3. 00	1. 70	1. 00

*In cholesterol-saturated medium.

SUMMARY

The passage of cancerous endocrine cells through culture and animal reveals that these cells can undergo alterations in their specialized properties. These alterations are stable but in most cases can be reversed.

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PLATE 14

FIGURE 1.—A pituitary tumor of the original animal passage line was disaggregated into single cells, and 10^7 cells per petri dish were plated. Two days later, the plates were washed to remove unattached cells, fixed and stained, and scored for attached and stretched cells. Less than 0.1 percent of the inoculated cells attached tc the petri dish surface had commenced growth (plate on *bottom*). A pituitary tumor derived from culture was plated in the same manner (plate on *top*). Approximately 100 percent of the inoculated cells became attached and stretched and commenced growth in culture.

714-852—64——12

MEMBRANE PERMEABILITY AND THE CONTROL OF CELLULAR FUNCTION [1]

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"MEMBRANE-ACTIVE" COMPOUNDS

"The most striking feature of the ultrastructural organization of the cytoplasm is the frequent occurrence of membranous components in various cell organelles, as well as in the ground substance of the cytoplasm. Membranes of various dimensions and different organization appear to represent a common and basic principle of organization in the cytoplasm." This quotation from a review by Sjöstrand (1959) aptly describes the importance of membranes in animal cells. Since membrane structures play such a major role, any modification in their properties will clearly have important consequences for cells and tissues. Different fat-soluble compounds may thus influence the metabolism of tissues in various ways by interacting with the lipide components of membranes. Willmer (1961) has presented a review of possible mechanisms of action of steroids in terms of the packing of different steroid molecules into membrane structures. Recently the fat-soluble vitamins were shown to be active in regard to membranes. Vitamin D, for example, is important in maintaining the structure of mitochondria (DeLuca, Reiser, Steenbock, and Kaesberg, 1960). It has been suggested that the first consequence of vitamin E deficiency may be free radical damage to the lipoprotein membranes of cells and their organelles; lysosomal enzymes released as a result of such damage may be responsible for muscle degeneration in vitamin E-deficient dystrophic rabbits (Zalkin, Tappel, Caldwell, Shibko, Desai, and Holliday, 1962). In our studies at the Strangeways Laboratory, we have found that the molecular structure of vitamin A is peculiarly adapted to enable the vitamin to interact with and to modify lipoprotein membranes. This property of the vitamin enables it to control certain metabolic and functional activities of tissues.

Relatively little is known about the hydrolytic and catabolic activities of tissues as compared with synthetic biochemical processes. However,

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tissue culture techniques are well suited for the study of catabolic processes (Lucy, 1963), and in this sphere the action of excess of vitamin A on embryonic cartilage *in vitro* provides a complex example of the far-reaching effects of the control of cellular function obtained by alterations in membrane permeability. The mechanism of action of vitamin A has been a topic of interest in our laboratory since Fell and Mellanby (1952) used the technique of organ culture to demonstrate that the vitamin acts directly on skeletal tissue and that its action is not mediated through a system such as the endocrine glands. Subsequent investigations on the biochemical changes associated with the morphological effects of excess of the vitamin on limb-bone rudiments in culture have led to the finding that a primary action of vitamin A in this system is on the lipoprotein membranes of cell organelles.

VITAMIN A AND CARTILAGE *IN VITRO*

The characteristic histological alteration observed on treatment of embryonic chick limb-bone rudiments with excess of vitamin A *in vitro* is loss of metachromatic staining of the cartilage matrix (Fell and Mellanby, 1952). This reduction in metachromatic staining is associated with a loss of hexosamine from the tissue. Thus, it was found that after 6 days' cultivation in a medium containing added vitamin A (10 IU/ml) the wet and dry weights and amino sugar content of cartilaginous limb-bone rudiments from the embryonic chick were half those of control rudiments on normal medium (Dingle, Lucy, and Fell, 1961). Analyses of the DNA and RNA content of the treated rudiments revealed that, compared with control cultures, the quantity of DNA was 80 percent while the quantity of RNA was 40 percent. At the same time, the vitamin depressed the rate of oxygen uptake but increased that of lactic acid formation. The interesting observation was also made that there was twice as much acid-soluble nitrogen in the used culture medium (plasma-embryo extract clot) of the vitamin A-treated explants as in that of the control medium. This observation suggested that the rudiments had a greater proteolytic activity in the presence of the vitamin. That a hydrolytic, and in particular a proteolytic enzyme, might be concerned in the changes produced in cartilage matrix under the influence of excess vitamin A was also indicated by the similarity between the effects on the intercellular material produced by the vitamin and by papain protease (Fell and Thomas, 1960).

The first indication that changes in membrane permeability are concerned in the mechanism by which vitamin A causes these alterations in the composition of embryonic cartilage was the finding that hypotonic treatment of normal cartilage leads to a loss of metachromatic staining closely similar to that produced by excess of the vitamin in culture (Lucy, Dingle, and Fell, 1961). It was found that embryonic chick cartilage contains an enzyme that is able to attack the matrix; this enzyme can be released from the chondrocytes by hypotonic treatment of normal car-

tilage in the cold. Subsequent incubation of the tissue in 0.1 M sodium acetate-HCl buffers of differing *p*H leads to a rapid loss of metachromatic staining of the matrix between *p*H 3 and 5, while there is little effect on metachromasia at *p*H 1, 2, 6, and 7. This loss of staining properties is associated with loss of half the hexosamine content of the rudiments; the hexosamine liberated is, however, mostly contained in polysaccharide of high molecular weight. In our experiments, it was observed that degraded protein components were released from the rudiments simultaneously with the liberation of the undegraded polysaccharide material. This observation, and the fact that an extract having proteolytic activity with an acid *p*H optimum was also obtained from normal cartilage, indicated that degradation of the protein moiety of the polysaccharide-protein complex was responsible for the dissolution of the matrix of normal cartilage treated in this way.

By analogy, it was thought that the degradation of cartilage matrix under the influence of excess of vitamin A *in vitro* might also result from the action of a proteolytic enzyme. It was suggested that the action of vitamin A on cartilage was mediated by a change in the permeability of the lysosomes of the chondrocytes, and that the protease thereby released subsequently attacked the cartilage matrix. This hypothesis was supported by the observation that treatment of isolated rat liver lysosomes with vitamin A results in liberation of a cathepsin-like enzyme (Dingle, 1961). The activity of such a proteolytic enzyme in cartilage could thus account for the observed loss of hexosamine from cartilage treated with excess of vitamin A *in vitro*, and also for the greater proteolytic breakdown of the culture medium in vitamin A-treated cultures (Dingle, Lucy, and Fell, 1961). The increased lactic acid formation observed in the treated cultures would naturally favor the action of an acid cathepsin. More recently, it has been shown that such rudiments do actually release an acid protease into the culture medium in the presence of excess vitamin A, and that a crude protease preparation derived from rat liver lysosomes effectively degrades the extracellular material of cartilage, even when the bulk *p*H of the culture medium is neutral (Fell and Dingle, 1963).

HEMOLYTIC ACTION OF VITAMIN A

In our attempts to elucidate the biochemical mechanisms by which vitamin A is able to act on membranes and hence to produce drastic changes in morphology and in the processes of differentiation, we followed a path that led us into several different fields and which resulted in the discovery of a number of hitherto unrecognized properties of the vitamin. One of these findings was that vitamin A alcohol is strongly hemolytic. The addition of a suspension of erythrocytes to vitamin A alcohol dissolved in ethanol causes rapid lysis of the cells and the production of red cell ghosts (Dingle and Lucy, 1962). Both the loss of hemoglobin, and the loss of potassium that precedes it, are inhibited by homologous serum;

bovine plasma albumin (fraction V) is also inhibitory. Thus it would appear that hemolysis of erythrocytes in animals suffering from hypervitaminosis A is probably prevented by the protective action of serum proteins in the blood. The ability of excess vitamin A, that is attached to serum proteins, to cause biochemical and morphological changes in tissues such as cartilage during cultivation *in vitro* is probably dependent on utilization of the protein by the cartilage tissue and simultaneous release of the vitamin from the carrier protein.

It was observed in our experiments with erythrocytes *in vitro* that the quantity of vitamin per cell was critical and, if the number of cells per unit volume of saline was reduced, less vitamin was required to give the same degree of lysis; this result suggested that the vitamin had a direct action on the cells. A further important aspect of the hemolytic action of vitamin A is that it is closely dependent on molecular structure and, with certain exceptions, the observed relationship between structure and activity is very similar to that for the action of vitamin A in animals. For example, of the substances tested on erythrocytes, no compound without both a β-ionone ring and a conjugated double-bond system had any effect on the red cells at a concentration of 35 μM. Hemolytic activity was reduced on increasing or decreasing the length of the side chain or on hydrogenation of the chain; oxidized vitamin A was inactive. The anhydro and hydrogenated derivatives of the vitamin, which are unable to prevent deficiency in animals, are also unable to produce the characteristic changes in cartilage caused by excess of vitamin A alcohol (Fell, Dingle, and Webb, 1962) or to hemolyze erythrocytes. It is interesting that despite the fact that vitamin A acetate is active in animals, and also in tissue culture (Fell and Mellanby, 1953), this compound had little hemolytic activity. The nature of the group at the end of the side chain is therefore important for hemolysis to occur. Dingle and Lucy (1962) suggested that the activity of ester derivatives normally observed depends on enzymic conversion to the free alcohol form.

How does vitamin A lyse erythrocytes? In studies made with phase-contrast and electron microscopes, we have seen that the initial effect, which occurs within 1 minute, is severe distortion of cellular shape and a greatly increased surface area relative to untreated cells (Glauert, Daniel, Lucy, and Dingle, 1963). It has been possible to follow the successive stages of hemolysis by use of different concentrations of the vitamin in combination with variations in the treatment temperature. The rapid increase in surface area leads to the formation of deep indentations in the surfaces of the cells (figs. 1 and 2). The subsequent early stages of lysis closely resemble the process of micropinocytosis in amoebae, especially the pinching off of small vacuoles from tubelike invaginations. The vitamin apparently causes local instabilities in the cell membrane; numerous breaks are visible in the membranes of the ghosts finally produced and hemoglobin might be lost at these points.

Schulman and Rideal (1937) showed that the hemolytic properties of many substances are paralleled by a capacity to penetrate a monolayer of

cholesterol at an air-water interface. In studies made at the Institute of Animal Physiology, Babraham, Cambridge, it was demonstrated that vitamin A also is a highly surface-active compound (Bangham, Dingle, and Lucy, unpublished observations). Vitamin A_1 alcohol is able to penetrate and increase the area of a monolayer of lecithin and cholesterol when the initial surface pressure of the monolayer is considerably greater than the collapse pressure of a film of the vitamin by itself. Unlike the structures of many closely related derivatives, the lipophilic and hydrophilic moieties of the molecule of vitamin A_1 alcohol appear to be balanced in such a way as to produce a very surface-active compound. Vitamin A acetate and the methyl ether of vitamin A are only weakly surface active; the activity of such derivatives *in vivo* and in tissue culture may well be the result of hydrolysis to the more active alcohol form of the vitamin. The relative inactivity of the esters no doubt protects the liver, where the vitamin is primarily stored, from gross tissue damage. We think that the molecular specificity observed in our experiments on the penetration and expansion of lipide monolayers may explain much of the molecular specificity observed in the biological effects of vitamin A. Penetration of vitamin A alcohol into lipoprotein membranes may be the initial step in many of the actions of the vitamin.

Massive penetration of vitamin A into a lipoprotein membrane might well be expected to alter the permeability properties of the membrane. Some indication of the mechanisms by which the permeability change actually occurs may be given by recent observations on the effect of saponin on the erythrocyte membrane. Treatment of red cells with saponin results in the production of a hexagonal array of "holes" 80 A in diameter which are visible in negatively stained preparations viewed with the electron microscope (Dourmashkin, Dougherty, and Harris, 1962). It has been shown that similar hexagonal patterns can be produced in synthetic mixtures of lecithin, cholesterol, and saponin in the absence of protein (Bangham and Horne, 1962: Glauert, Dingle, and Lucy, 1962). The structures observed in red cells treated with saponin are apparently the result of a rearrangement of the lipides of the cell membrane, and the aqueous areas, 80 A in diameter, thus produced would easily allow the escape of hemoglobin from within the cell. Further studies of the structures of complexes of cholesterol and saponin, and of lecithin, cholesterol, and saponin, have shown that each complex exists in specific lamellar and hexagonal phases (Lucy and Glauert, 1963). It is possible that specific rearrangements of membrane lipides may also occur on treatment with vitamin A. Although the rapid lytic action of vitamin A alcohol probably results from oxidation of the vitamin after it has penetrated the lipoprotein membrane of the erythrocyte, since rapid lysis is inhibited by vitamin E, our experiments also indicate that α-tocopheryl acetate used in conjuction with vitamin A alcohol does not prevent vitamin A from penetrating the membrane (Dingle and Lucy, 1963). Under physiological conditions, specific lipoprotein structures intimately concerned with permeability may thus result from the presence of the two fat-soluble vitamins together

within membranes, especially if vitamin A is concerned in the binding of lipide to protein in membranes (Lucy and Dingle, 1962).

VITAMIN A AND INTRACELLULAR PARTICLES

Although the molecular specificity for the action of vitamin A on membranes is high, there appears to be much less specificity regarding the type of membrane affected by excess of vitamin A. Addition of the vitamin to rat dermal fibroblasts in buffered saline, in the absence of protein, causes local distensions of the cell membrane which finally disintegrates (Dingle, Glauert, Daniel, and Lucy, 1962). The nuclear membrane also may be damaged, although it is sometimes still recognizable when the cell membrane has completely disappeared. The refractile granules seen in living cells enlarge or coalesce to form large dense bodies; these bodies are probably lipide granules and in thin sections they sometimes have a complex structure with a dense center surrounded by lighter membrane material. The mitochondria of these cells treated with vitamin A became swollen and finally lost their cristae. At the same time, the cisternae of the endoplasmic reticulum became distended and filled with granular material. The ribonucleoprotein particles remained attached to the outer surfaces of the cisternae, however, throughout the disintegration of the cell (figs. 3 and 4). Fitton Jackson and Fell (1963) in a study on the epidermal fine structure of embryonic chick skin under the influence of excess vitamin A *in vitro* have observed changes in mitochondria. After 6 or 7 days' cultivation, the mitochondria enlarged, the increase in size being greater in the basal cells than in the superficial layers; the internal cristae continued to project inward at right angles to the surface, but the mitochondrial sap became electron-lucent and greatly increased in amount. In experiments with isolated subcellular particles, it has been shown that vitamin A alcohol causes rapid release of hydrolytic enzymes from lysosomes (Dingle, 1961) and that, like thyroxine, the vitamin is able to cause mitochondrial swelling *in vitro* at concentrations similar to those of the hormone (Lucy, Luscombe, and Dingle, 1963). The idea that the initial action of vitamin A on membranes depends on reaction of the vitamin with lecithin and cholesterol is consistent with the wide spectrum of membranes affected by excess of the vitamin, although the final effect of the vitamin will clearly depend on the particular membrane concerned.

CONCLUSIONS

Our observations that vitamin A acts on membranes are of interest in themselves since they indicate a mechanism by which excess vitamin A, and possibly also deficiency of the vitamin, affects the biochemistry and

physiology of tissues. Indirect control of enzyme action and of the extracellular components of tissues are among the results of the action of the vitamin on membranes. By implication, however, our findings are also of wider interest since they provide experimental evidence, with one particular compound, for the existence of a control mechanism in the cell which may operate with lipide-soluble substances such as hormones and the other fat-soluble vitamins. These compounds when present in only microgram quantities have far-reaching effects on the behavior of tissues. I should like to suggest that substances of biological importance shown to act in this way may be suitably described as "membrane-active." One striking feature of the action of vitamin A *in vivo* is that it affects the functional activities of so many different parts of the body. It is not implied that the action of the vitamin is solely concerned with membranes; nevertheless, our experiments have shown that the vitamin has a widespread effect on membranes, and since membranes are such an important feature of cellular and tissue architecture, it is conceivable that this action of the vitamin in animals may account for the broad syndrome of its effects.

SUMMARY

Vitamin A is peculiarly adapted for interacting with lipoprotein membranes, and this property is closely dependent on the precise molecular structure of the vitamin. Thus, compounds such as anhydro and hydrogenated vitamin A, that are inactive in preventing vitamin A deficiency and are also unable to produce the characteristic changes of hypervitaminosis A, have relatively little action on lipoprotein membranes. In contrast, vitamin A alcohol is able to penetrate the membrane of the isolated erythrocyte with great rapidity and produce changes consistent with an increase in the surface area of the cell. These changes lead to rapid hemolysis of the erythrocyte. The hemolytic action of vitamin A alcohol is paralleled by its ability to penetrate and expand the area of a monolayer of lecithin-cholesterol at an air-water interface.

The action of vitamin A on membranes is not restricted to the plasma membrane of the erythrocyte. For example, the hypervitamonic changes produced in cartilage appear to result primarily from the action of the vitamin on the lysosomes and mitochondria of the cartilage cells. In cultures of cartilage treated with the vitamin, a protease with an acid *p*H optimum is released from the lysosomes; this protease subsequently attacks the protein-polysaccharide complex of the intercellular matrix and thereby produces the characteristic loss of metachromatic staining. At the same time oxygen uptake is diminished, while lactic acid production is increased, thus favoring the action of acid hydrolases. It is thought that one of the normal sites of action of vitamin A is within membranes, and that control of cellular function by the vitamin may result from its effect on the properties and the stability of structural lipoprotein systems.

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PLATE 15

Electron micrographs of thin sections of rabbit erythrocytes

FIGURE 1.—Normal cells. × 8,000

FIGURE 2.—Thirty seconds after addition of the cells to vitamin A alcohol (10 μg/ml) at 25° C. Deep indentations are visible in cell surfaces. × 8,000


1

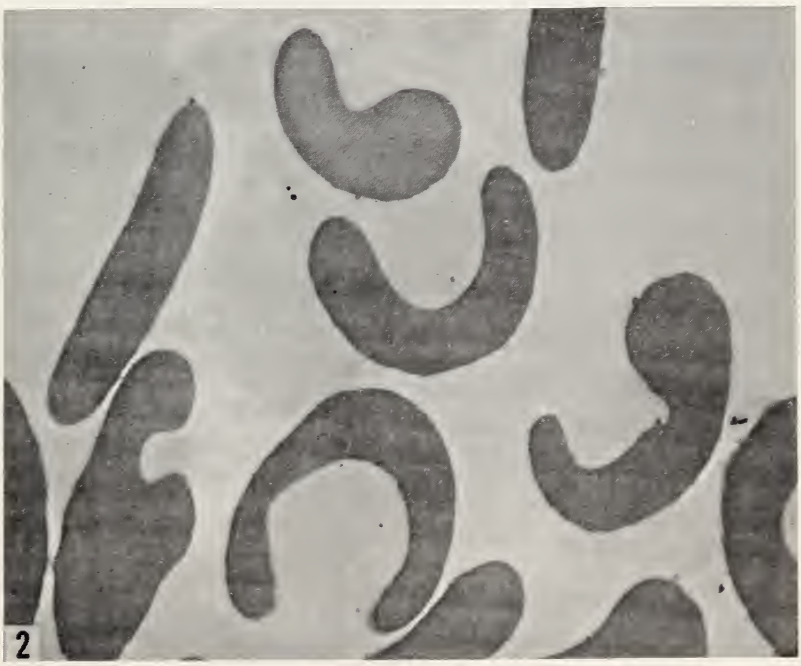

2

PLATE 16

FIGURE 3.—Electron micrograph of a thin section of a rat dermal fibroblast from a saline suspension. Cell membrane appears as a continuous dense line, elongated profiles of the cisternae of the endoplasmic reticulum are present, and the mitochondria are small and dense. The nucleus and Golgi zone appear normal. Empty pinocytotic vacuoles and small lipide droplets are present. × 18,000

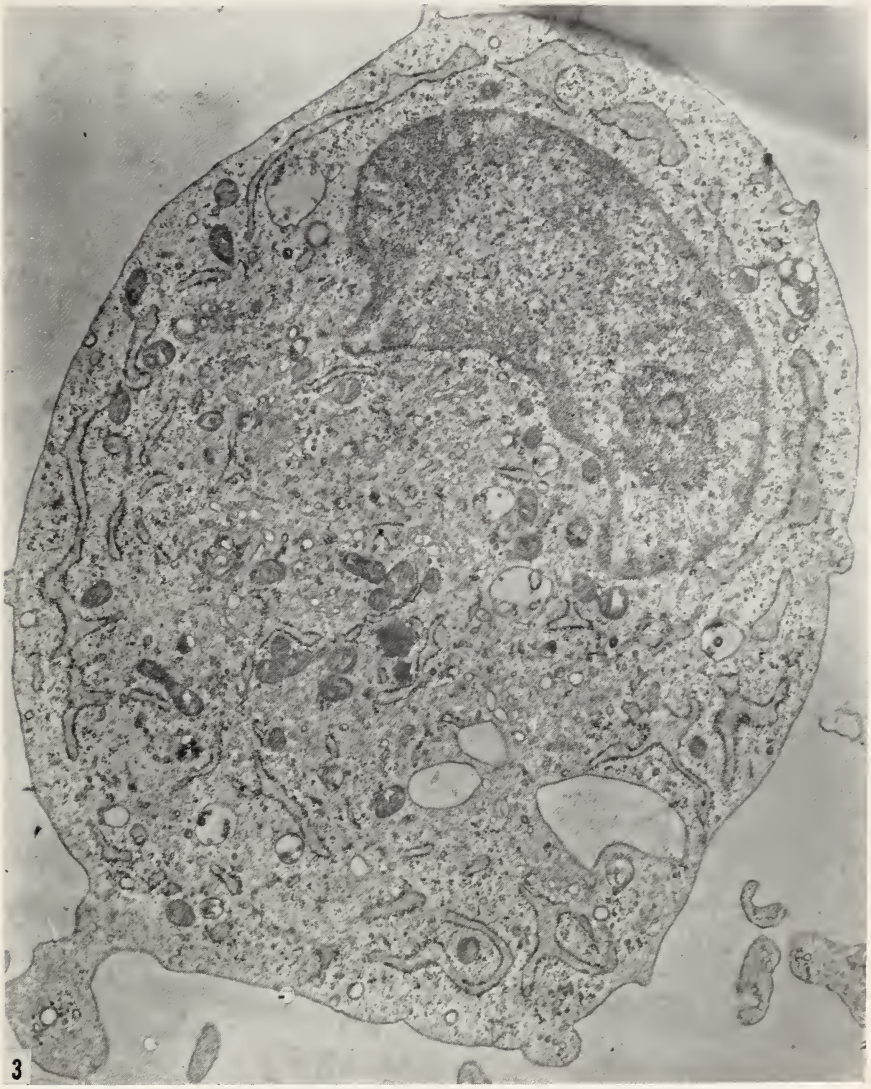
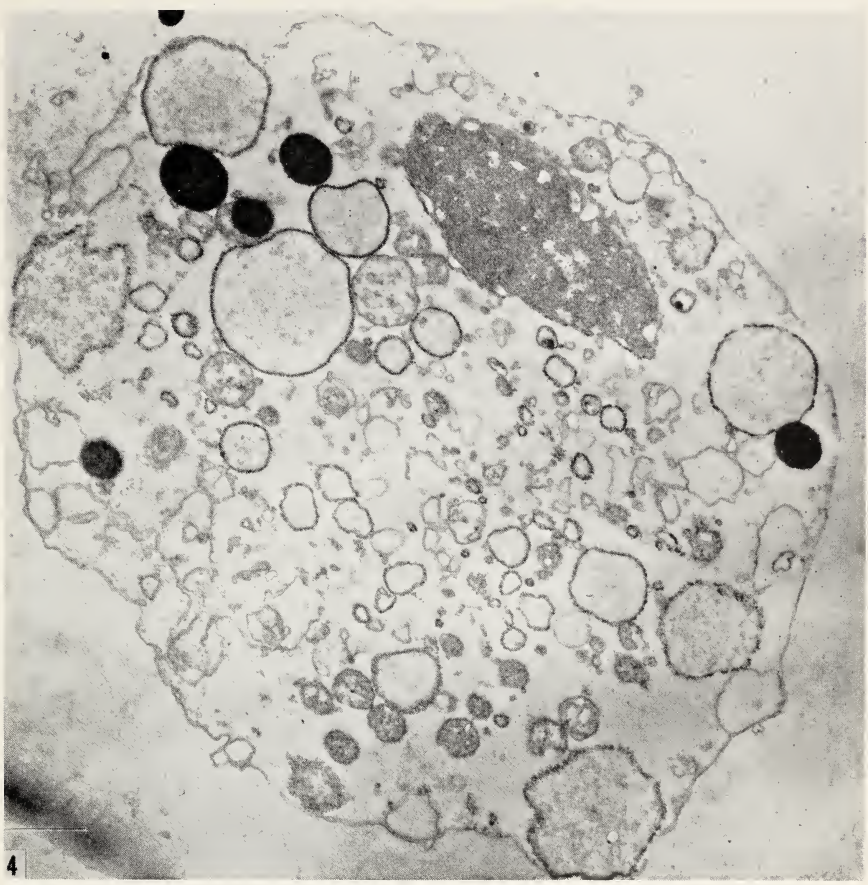

3

PLATE 17

FIGURE 4.—Electron micrograph of a thin section of a rat dermal fibroblast incubated in the presence of vitamin A alcohol (20 μg/ml) for 10 minutes at 37° C. Cell membrane is broken, cisternae of the endoplasmic reticulum and the mitochondria are swollen. Nucleus appears damaged and lipide droplets are greatly enlarged. $\times$ 18,000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ACTION OF NUCLEIC ACIDS

Chairman: Van R. Potter

714-852—64——15

MODELS AS AIDS TO COMMUNICATION [1]

VAN R. POTTER, Ph.D., McArdle Memorial Laboratory,
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I was asked by Dr. Rutter to make some "general remarks" to open this session, and I understood that I should try to avoid the esoteric meanings of our current research. In a group as large and diverse as the Tissue Culture Association, and in a session devoted to the function of the nucleic acids, there must be a variety of mental images that are conjured up when someone mentions the letters DNA or the popular term "messenger RNA." This variety of images includes not only the molecules themselves but also the concepts by which they are functionally related to each other.

I was recently impressed on rereading Lewis Mumford's *Values for Survival* by his remarks (pp. 200–220) about the usefulness of symbolic devices: "To make up for the increasing bulk of factual material, science in particular provides a system of shorthand notation, in the form of laws, or in the form of symbolic devices, like maps in geography. . . . Thanks to these notations and symbols, those familiar with their use can see the view from the mountain top without having to climb every foot of the way; thanks to works of art, the experience of a multitude of lives can be utilized within a single lifetime. . . . What we need is not some all-embracing encyclopedia, which will be out of date at many points before it is even published; we require, rather, the invention of conceptual devices, and the perfection of a discipline, which will progressively transform our unrelated fragments of experience into an intelligible whole." I hope that the inclusion of the words "and the perfection of a discipline" makes it clear that both Mumford and I have as high a regard for methodology and technique as we have for conceptual devices.

It is becoming clear that in seeking "conceptual devices" we are looking for a shorthand notation that we can all read. We are asking what is the minimum body of knowledge we all must have in order to plan and carry out our experiments effectively and to communicate with each other and the world.

In the development of these conceptual devices we are making more and more use of molecular models. But what is a model? Do the molecules

[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27–30, 1963.

really look like the models or is the model simply a product of the imagination? Or is it an abstraction or image that we use as a tool for thinking and communicating with each other? In this new age of molecular biology, it is clear that communication and thinking cannot be accomplished with words alone, except in so far as the words become symbols for the models which are the images of the substances we are talking about.

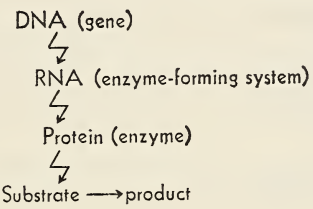
Kenneth Boulding, Professor of Economics at the University of Michigan, has written a remarkable book called *The Image*, which I think may help to put our models and diagrams in perspective and help us to accept them for what they are—not the embodiment of ultimate knowledge but as a means of communication: "Knowledge has an implication of validity, of truth. What I am talking about is what I *believe* to be true; my subjective knowledge. It is this *Image* that largely governs my behavior" (pp. 5–6). "Images can never be compared with any outside reality. . . . *Images* can only be compared with *images*" (p. 165). "What we mean by truth, or at least the progress toward truth, is an orderly development of the image, especially of the (shared) and (recorded) image, through its confirmation by feedback messages" (p. 169.) In these terms "messages consist of information in that they are structured experiences. The meaning of a message is the change which it produces in the image" (p. 7).

But "Our message input depends to a considerable extent upon our existing value structure" (p. 174).

With these thoughts in mind it is interesting to look at some models that represent the available means of communication between tissue culturists, enzymologists, and molecular biologists. As we all know, DNA is the icon of the molecular biologists and Francis Crick and Jim Watson are the prophets who first conceived the 3-dimensional model, now widely understood at various levels of sophistication. It may be interesting to look at the many forms these models have taken. As we compare "images with images," we each have a mental reaction relating the model to our own image of the actual molecules, whether we are looking at the bent-wire spirals, the ball and stick atoms that indicate the principle but not the space-filling properties of the constituent atoms, or the models with both angles and atomic diameters rendered as accurately as possible in terms of modern chemistry. Finally there is the do-it-yourself model that I developed in 1959 when I constructed 3-dimensional models of molecules from 2-dimensional atoms using the conventional and familiar 2-dimensional notations of the organic chemist. From these various 3-dimensional models in the realm of common knowledge, it is possible to communicate in 2-dimensional notations on slides or

blackboards with parallel rows of opposed sequences of the letters A, G, C, and T lined up according to the Watson-Crick rule of A opposite T and G opposite C, and to discuss RNA sequences using the notation pApCpGpU. Thus when the letters DNA and RNA appear in a symposium a variety of images must be lighted up in our separate minds.

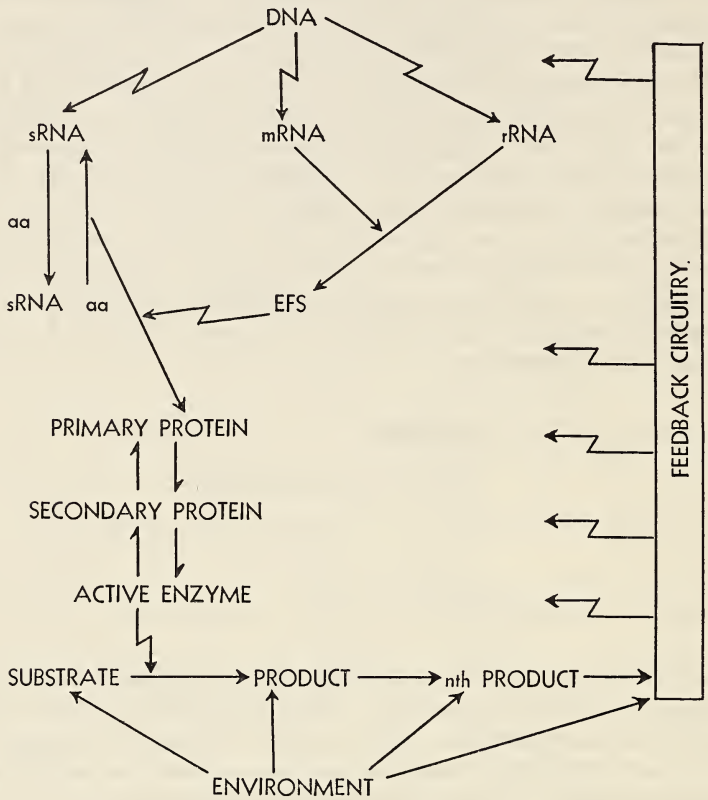
As to the functional relations between DNA and RNA, we may refer to the Central Dogma formally, or perhaps informally, stated by Crick, in 1958, that information flows from nucleic acid to nucleic acid or from nucleic acid to protein but not from protein to nucleic acid. Many times we have seen chemists or molecular biologists drawing arrows from DNA to RNA to indicate this flow of information, ignoring the fact that for years a straight arrow between two substances A and B has been translated by chemists to mean "A is converted to B." Therefore, I have attempted to introduce the use of a jagged arrow to distinguish information transfer, or control functions from conversions, with the result that the simplest form of the Central Dogma has appeared in the form of the following image or ideogram:



This has been used to indicate the basic units that replace the cell as the foundation of all living organisms. This arrangement implies that information does not flow from RNA to DNA, though the original statement of the Dogma left that question open, and negative evidence does not constitute proof. There is, however, wide agreement on the generality of this formulation, and if there are any instances in which information in RNA directs DNA synthesis, they remain to be demonstrated.

Meanwhile, rapid progress in the dissection of the enzyme-forming system has indicated at least 3 kinds of RNA, and we now must rewrite the diagram to include the amino acid transfer RNA or soluble RNA (sRNA), the ribosomal RNA (rRNA), and the messenger RNA (mRNA). We must also recognize the association and dissociation of the primary proteins into secondary proteins of higher molecular weight, and the reversible conversion of fully formed proteins into active enzymes by

interaction with smaller molecules at "allosteric" or regulatory sites. Finally, it appears that there are feedback loops from the metabolic products or from the environment to control points that appear to be potentially available at every level:



It is apparent that the more recent image or model of the basic unit of life represents a considerable advance over the first, since it provides for considerable modulation of gene activity by intracellular and extracellular feedback mechanisms, whereas the first diagram gave no clue that the expression of the information in DNA molecules is subject to modulation. The second diagram also enables us to pinpoint the molecular sites of various agents blocking enzyme action or enzyme synthesis. It appears that actinomycin D is a useful agent for blocking DNA-directed RNA synthesis, while puromycin appears to block the incorporation of amino acids into new protein.

There are a number of unanswered questions, of which two are the following:

1. What determines whether a given messenger RNA is to have a long survival (days, weeks, or months) or whether it is to survive only a few minutes or hours? At present actinomycin appears

to be an aid in answering this question in that a continuing enzyme synthesis in the presence of actinomycin is taken to mean that the messenger RNA is stable for the period of observation.

2. Does all RNA represent transcription of DNA, or is RNA-directed RNA polymerase functionally important in some systems? Data so far available suggest that this polymerase may be important only in the case of certain RNA viruses.

Studies on RNA synthesis in relation to protein synthesis strongly suggest that there are critical times when blocking synthesis of RNA blocks protein formation, followed by periods when protein synthesis is insensitive to RNA blockers.

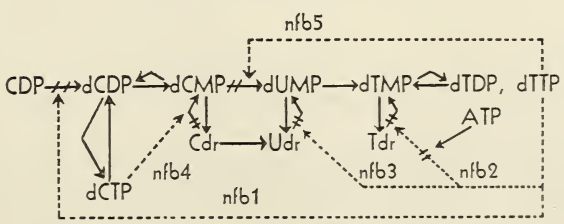
But as we look at the current image or model of the basic unit of life, we realize that to this picture we must add the role of the membranes within cells and the membranes between cells.

For the product of one enzyme may be the inducer or repressor for the gene responsible for another enzyme not only in the same cell but in another cell, adjacent or remote, and the intervening membranes may play an important role in the outcome. In the current discussion of regulator genes acting on operator genes that control structural genes it may be helpful to assume, for the moment, that the regulator gene is merely a structural gene for a product entering the feedback circuit. What is needed is the identification of the products and their exact molecular targets.

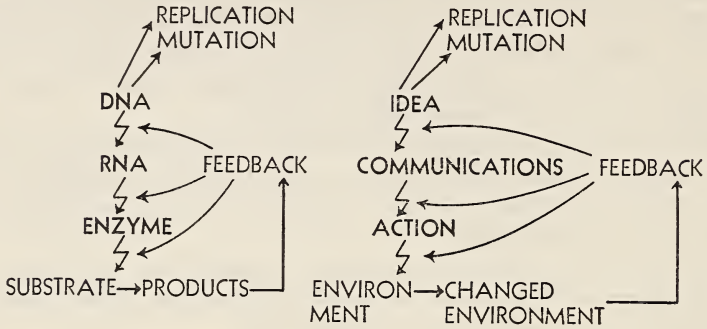
Here we may note that the rapid adjustments in cell physiology probably do not involve changes in enzyme synthesis but do involve changes in enzyme activity, through association and dissociation of protein molecules, through regulator sites, and through conventional forms of activation and inhibition.

Biochemists are mapping not only the metabolic pathways, but the feedback pathways as well. An example is pyrimdiine biosynthesis, studied to some extent in my laboratory, and more extensively by Maley and Maley whose recent results are published in this Monograph in which they provide data in support of a regulator site on dCMP deaminase, which is *activated* by dCTP. Thus to the present charts of metabolic pathways and negative feedback pathways a third network must be added to represent activating pathways.

NEGATIVE FEEDBACK
IN BIOSYNTHESIS OF dTTP



Finally I would like to emphasize the analogy between *ideas* and DNA molecules by means of the following diagram.



The analogy is probably a valid one because both DNA molecules and ideas represent information that can be expressed in action, and is subject to feedback, replication, mutation, and recombination. In this chart, the *subject* of our symposium is on the *left*, and the *process* of our symposium is on the *right*. We hope that there will be further discussion of the feedback process.

THE REGULATION OF DEOXYRIBONUCLEIC ACID SYNTHESIS IN EMBRYONIC TISSUE[1, 2]

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The cell as a unitary organism or as part of a social order can survive only by the ability to reproduce itself. The manner in which this process is accomplished was shown to be intimately related to the information derived from pre-existing DNA[4] and the control processes regulating the presentation of this information to the organism (*1*). In recent years, mainly through the studies of Kornberg and his associates (*2*), a host of knowledge has been accumulated concerning the structure of DNA and the mechanisms involved in the replication of this molecule. However, before DNA can be replicated, it must be presented with its basic precursors in the presence of DNA polymerase. It is apparently the rate of synthesis of these precursors, in particular dTMP, that regulates and possibly triggers the synthesis of DNA. The proposal that dTMP synthesis is a regulatory factor in DNA synthesis has come from numerous studies demonstrating either the absence or limited presence of enzymes associated with the synthesis and final conversion of this nucleotide to thymidine triphosphate (*3–7*) in nongrowing as compared to mitotically active tissues. It might be anticipated that on the induction of cell division these enzymes would become less limiting and thus would be vital in providing an essential substrate for the synthesis of DNA.

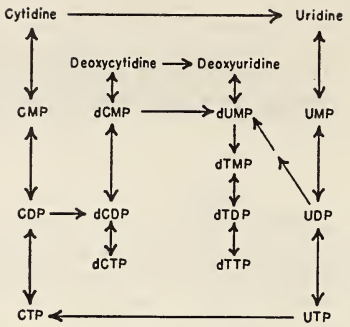
To better understand the metabolic routes available for the synthesis of dTMP from its basic precursors, a diagram of the known and proposed reactions involving these compounds is presented in text-figure 1. As demonstrated by Lieberman (*8*) and Kammen and Hurlbert (*9*), UMP is converted to CTP in a DON-sensitive reaction involving glutamine. The corresponding deoxyribonucleotide transformation apparently occurs at the ribonucleoside diphosphate stage (*10*, *11*) with the products feeding into dTMP as indicated.

[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27-30, 1963.

[2] Supported in part by research grant C-06406 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

[4] The abbreviations used are: DNA, deoxyribonucleic acid; d-, deoxy; DON, 6-diazo-5-oxo-L-norleucine; CMP, CDP, CTP, cytidine 5′-mono-, di-, and triphosphate, respectively; dTMP, dTDP, dTTP, thymidine 5′-mono-, di-, and triphosphate, respectively; UMP, UDP, UTP, uridine 5′-mono-, di-, and triphosphate, respectively; dGMP and dGTP, deoxyguanosine 5′-mono-, and triphosphate, respectively; dAMP and dATP, deoxyadenosine 5′-mono-, and triphosphate. respectively.



TEXT-FIGURE 1.—Pathways of cytidine and uridine metabolism in chick embryo.

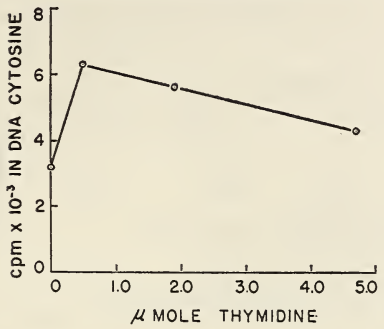
Our studies in this area stemmed from the discovery of deoxycytidylate deaminase in embryonic tissue (*12*, *13*) and subsequent attempts to clarify its suggested role in DNA synthesis. As indicated in text-figure 1, the conversion of dCMP to dUMP could provide an important link between the cytidine nucleotides and dTMP, perhaps a vital one if the synthesis of dTMP via the uridine⟶UMP⟶UDP⟶dUDP⟶dUMP pathway is rate-limiting. However, the existence of such a role for deoxycytidylate deaminase has been by implication only, except for a few studies demonstrating the elevation of this enzyme in mitotically active tissues (*7*, *14*, *15*) and phage-infected cells (*16*). Unfortunately though, the contribution of this enzyme to the over-all DNA synthetic pathway could not be quantitatively assessed from such studies. Although our studies presented in this report still do not provide a direct answer to this problem, they shed new light on factors that may control the metabolism of d-cytidine through their influence on deoxycytidylate deaminase.

RESULTS AND DISCUSSION

Mince Studies

The finding that dTMP (or a derivative) could inhibit the deaminase *in vitro* (*12*) suggested the possibility that the presentation of an excess of this compound to a tissue would inhibit the deaminase *in vivo* and alter the incorporation of a labeled precursor such as dCMP-2-C^{14} into the DNA. For these studies, 4-day chick embryo mince was selected because of the availability of the tissue and the rapid incorporation of labeled precursors into the DNA.

As indicated in text-figure 2, this study revealed that thymidine enhanced the incorporation of dCMP into the cytosine of the DNA about twofold, at the lowest concentrations of thymidine employed (0.25 mM). At higher levels the incorporation rate progressively decreased, until at the 2.5 mM level it was only slightly above the control. Thymine did not duplicate the results of thymidine. The acid-soluble fraction revealed a decrease in the specific activity of the isolated deoxyuridine (table 1)



TEXT-FIGURE 2.—The incubation mixtures contained 0.8 g 4-day chick embryo mince; dCMP-2-C^{14}, 0.39 μmole (3.91×10^5 cpm/μmole); or cytidine-2-C^{14}, 1.84 μmole (2.77×10^5 cpm/μmole); the additions, 0.50 μmole, Krebs-Ringer phosphate to 2.0 ml. Incubation time, 2 hours at 37° C. *See* (*18*) for methods.

with increasing thymidine concentration and an increase in the total amount of d-cytidine that could be isolated. While the decrease in the specific activity of d-uridine in this experiment may have been due to a contamination with uracil, 2-dimensional chromatography (*17*) of the isolated nucleotide fraction, which effectively separates these compounds, yielded similar results. The relationship between these results and the enhancement of the cytosine specific activity is not entirely clear at present and will be discussed in more detail later, but it does suggest that more d-cytidine is available for incorporation into DNA cytosine in the presence of thymidine than in its absence.

An explanation for this effect can be derived from the likely possibility of a stimulation in the synthesis of DNA in the presence of thymidine, but this can be excluded on the basis of the Pi^{32} incorporation studies presented in table 2. Thymidine alone did not affect the incorporation, but in the presence of d-cytidine an inhibitory effect was observed. The inhibition was enhanced still further by d-adenosine, which has been shown to be an inhibitor of chick embryo DNA synthesis (*20*), and was overcome by the addition of d-guanosine. These effects are probably related to the inhibition of the ribose→deoxyribose conversion involving the guanosine nucleotides, and has been discussed more completely by Klenow (*21*). However, most pertinent to the findings described in this report is that an increase in DNA synthesis was not observed with thymidine plus

TABLE 1.—Nucleosides isolated from dCMP-2-C^{14} incubation with chick embryo*

Thymidine added (μmoles)	Nucleosides plus bases (cpm)	d-Cytidine (cpm)	d-Uridine	
			(cpm)	(cpm/μmole)
0	89, 400	33, 750	48, 100	1.96×10^5
0. 47	48, 800	68, 500	25, 800	1.30×10^5
1. 88	46, 000	63, 450	17, 750	0.94×10^5
4. 70	42, 200	75, 850	17, 100	0.65×10^5

*0.46 μmole dCMP-2-C^{14} (3.91×10^5 cpm/μmole) and 0.9 g of chick embryo mince. *See* (*15, 19*) for methods.

TABLE 2.—Incorporation of Pi^{32} into chick embryo DNA

Additions	DNA (cpm/mg)	
None	63, 500*	8, 390†
Thymidine‡	66, 800	8, 530
d-Cytidine	49, 000	6, 500
d-Cytidine, thymidine	51, 400	5, 820
d-Cytidine, thymidine, d-adenosine	45, 000	
d-Cytidine, thymidine, d-adenosine, d-guanosine	59, 400	

*Incubation of chick embryo mince in 0.9 percent NaCl.
†Incubation of chick embryo mince in Krebs-Ringer phosphate.
‡Concentration of the added nucleosides was 0.25 mM. *See* (*20*) for methods.

d-cytidine under conditions analogous to those described for dCMP-2-C^{14} in text-figure 2.

Upon enzymatic hydrolysis of the Pi^{32}-labeled DNA, the isolated dTMP possesses the highest specific activity (table 3) of the four deoxynucleotides in the DNA, which suggests that the thymidine nucleotide pool is probably the smallest of the four. Similar observations by Langen and Liss (*22*) are in accord with the proposal that dTMP synthesis may regulate the rate of DNA synthesis.

Another explanation for our results (text-fig. 2) is that thymidine (or most likely a phosphorylated derivative) inhibits the conversion of CDP to dCDP. A similar effect has been described by Morris and Fischer (*23*) in studies with ascites cells, and such a block would essentially reduce the dilution of exogenously supplied d-cytidine derivatives by that produced *de novo*. The net effect would be a higher intracellular specific activity of the experimentally supplied labeled derivative in the presence of thymidine, and result in an apparent increase in the rate of incorporation. That this does not apply in this instance is demonstrated in table 4, where it is shown that thymidine does not impair the incorporation of cytidine-2-C^{14} into DNA cytosine. It may be questioned as to why an enhancement of incorporation was not observed, but as seen in text-figure 1, cytidine enters the d-cytidine pool at the diphosphate stage and

TABLE 3.—Incorporation of Pi^{32} into chick embryo mince DNA

Experiment	DNA (counts/min/mg)	Nucleotides isolated from DNA (counts/min/μmole $\times 10^{-4}$)			
		dTMP	dGMP	dAMP	dCMP
I	128, 600	4. 20	3. 49	2. 88	1. 90
II	242, 000	8. 30	6. 35	5. 48	3. 65
III	236, 000	7. 55	6. 45	4. 85	3. 19

The experimental conditions are similar to those described in (*20*). Incubation was at 37° C for 2 hours. The specific activity of Pi^{32} was: I, 2.8×10^7; II, 4.0×10^7; and III, 4.5×10^7 counts per minute per μmole. After isolation of DNA, it was digested with DNase and phosphodiesterase and the 5′-deoxynucleotides were isolated by chromatography.

TABLE 4.—Incorporation of dCMP-2-C^{14} and cytidine-2-C^{14} into chick embryo DNA*

Additions	dCMP-2-C^{14}		Cytidine-2-C^{14}	
	Cytosine (cpm/μmole)	Thymine	Cytosine (cpm/μmole)	Thymine
None	4, 750	7, 790	4, 480	1, 940
Thymidine	7, 300	856	4, 400	114
Thymidine d-Adenosine d-Guanosine	5, 550	106	2, 620	110

*See text-figure 2 for methods.

is not as readily subject to the initial reactions encountered by d-cytidine. These latter reactions are most probably responsible for the enhanced incorporation in the presence of thymidine. The proximity of dCDP to the final reactions involved in DNA synthesis would also explain why cytidine is incorporated predominantly into the cytosine of the DNA, whereas d-cytidine is directed mainly into the thymidine pool.

Incorporation Studies in the Presence of DON

To evaluate the extent to which cytidine nucleotides contribute to the d-cytidine pool, the incorporation of dCMP-2-C^{14} into DNA in the presence and absence of DON was determined. It was anticipated that the DON study would present a measure of the dilution of the d-cytidine nucleotide pool by precursors from the endogenous ribonucleotide pool and also answer the question whether added thymidine inhibits the cytidine→d-cytidine transformation. As seen in table 5, DON raises the specific activity of DNA cytosine to a level comparable to that obtained with thymidine, which suggests that the two compounds may achieve the same effect (the inhibition of dCDP formation) by acting at different sites. Although the two compounds gave similar results, they do not necessarily act in the same manner. Thymidine could yield the same effect by enhancing the efficiency of incorporation of d-cytidine nucleotides into DNA. The simultaneous presence of thymidine and DON in the reaction vessel yields an even greater specific activity in the

TABLE 5.—Effect of thymidine and DON on the incorporation of dCMP-2-C^{14} into chick embryo DNA*

Additions	Amount added (μmoles)	DNA (cpm/mg)	Cytosine (cpm/μmole)	Thymine
None	—	8, 010	4, 430	6, 460
Thymidine	0. 46	4, 840	5, 420	1, 030
DON	1. 00	8, 530	5, 710	5, 470
Thymidine + DON	0. 46 1. 00	5, 010	7, 840	848

*See text-figure 2 for methods.

TABLE 6.—Effect of DON on uridine-2-C^{14} incorporation into chick embryo DNA*

Additions	Amount added (μmoles)	DNA (cpm/mg)	DNA Cytosine (cpm/μmole)	DNA Thymine (cpm/μmole)
None	—	1, 300	514	1, 181
DON	1. 0	1, 262	0	1, 400
DON + d-Cytidine	1. 0 2. 0	1, 120	0	1, 245

*The incubation mixtures contained 0.9 g 4-day chick embryo mince, 1.6 μmole uridine-2-C^{14} (2.85×10^5 cpm/μmole), and additions as indicated. *See* text-figure 2 for methods.

cytosine of the DNA, which indicates that the added thymidine may have either increased the efficiency of the DON block, or, if acting by increasing the efficiency of dCMP incorporation into DNA, is doing so with nucleotides of a higher specific activity, as a result of the DON block. The latter would have the effect of increasing the specific activity of the DNA cytosine above that of DON alone and is the obtained result. Since the DON block, demonstrated in table 5, is complete by measuring the conversion of uridine-2-C^{14} to DNA cytosine (table 6), apparently thymidine does not act by increasing the efficiency of the DON block. The absence of an appreciable dilution effect in the presence of d-cytidine is difficult to explain and requires further clarification, since previous studies (*20*) in the absence of DON revealed that d-cytidine imposed a much greater dilution effect on the incorporation of uridine.

The 15 to 20 percent decrease in the specific activity of DNA thymine produced by DON (table 5) might be explained by an elevation in the uridine nucleotide pool due to the cytidine block, which could be expected to yield a slightly greater conversion of UDP⟶dUDP.

Phosphorylation of d-Cytidine in Extracts

It was hoped that *in vitro* studies on the products resulting from the phosphorylation of d-cytidine, in the presence and absence of thymidine derivatives, might explain the enhancement effect of thymidine on the incorporation of dCMP-2-C^{14}. Since a similar effect was observed with rat embryo mince (*19*), extracts of this tissue were used in preference to chick embryo extracts because of the higher content of phosphorylating enzymes in the former tissue. The experimental techniques and results are described in greater detail elsewhere (*19*), but essentially involve the reaction of d-cytidine-2-C^{14} or d-cytidine-H^3 with ATP, followed by acid hydrolysis of the deoxynucleoside polyphosphates to the monophosphate level and chromatographic isolation of the products. Table 7 presents a time study of d-cytidine-2-C^{14} phosphorylation revealing dUMP as the major product formed in the absence of added dTMP. However, the predominant product formed, in the presence of this compound, was d-uridine. Since dUMP can be formed by two pathways,

d-cytidine⟶d-uridine⟶dUMP (1)

d-cytidine⟶dCMP⟶dUMP, (2)

TABLE 7.—Deoxycytidine-2-C^{14} utilization by rat embryo extract in the presence of ATP

Time (min)	Amount converted to		
	dCMP (mμmoles)	dUMP (mμmoles)	Deoxyuridine (mμmoles)
20	3. 46	4. 23	0. 16
40	6. 37	9. 75	3. 16
60	7. 38	12. 10	4. 46
60 (+dTMP)	7. 00	3. 00	14. 10

Each reaction mixture contained 0.365 μmole of substrate (1.45×10^5 cpm/μmole). The enzyme source was an extract from 12- to 14-day embryos. The compounds were determined after acid hydrolysis of the reaction mixture. For details, *see* (*19*).

we investigated the nature of this apparent dTMP block by studying the conversion of d-uridine to dUMP in the presence of thymidine and related compounds. Table 8 shows that dTTP is by far the most effective and specific inhibitor when a comparison is made with other deoxynucleoside triphosphates. That the observed effect of dTMP in table 7 was due primarily to its conversion to dTTP is demonstrated in table 9, where a marked inhibition of dUMP formation from d-cytidine is observed. The apparent inhibition of dCMP formation by dCTP is not in reality an inhibition but, as will be shown, is due to an activation of dCMP→ dUMP conversion.

Since the d-uridine kinase reaction [5] is markedly inhibited by dTTP, we believed that a measure of dUMP formation by reaction sequence (2) (p. 122) could be obtained by studying it in the presence of dTTP. The absence of dUMP formation (table 9) indicates either that this compound is formed only by reaction sequence (1), or that deoxycytidylate deaminase is also inhibited by dTTP. That the latter is true is seen in table 10, which shows data obtained with chick embryo extract as the enzyme source (*18*). However, similar results have been obtained with rat embryo extracts (*19*). The inhibition, as with d-uridine kinase, reveals a distinct specificity (table 11). In contrast to the results obtained with dTTP, dCTP activates the deamination and would in part explain the apparent absence of dCMP formation in table 9.

TABLE 8.—Effect of deoxynucleoside triphosphates on deoxyuridine-H^3 phosphorylation*

Additions	Amount added (μmoles)	dUMP formed (μmoles)
None		99. 8
dCTP	0. 40	103. 0
dGTP	0. 15	93. 2
dATP	0. 38	98. 0
dTTP	0. 16	1. 71

*For substrate concentration and assay procedure, *see* (*19*).

[5] The reaction may be synonymous with thymidine kinase, as this enzyme catalyzed reaction has also been shown to be inhibited by dTTP (*24*).

TABLE 9.—Deoxycytidine-H^3 utilization in the presence of deoxynucleotides*

Additions	Amount added (μmole)	Compound formed: dCMP (mμmoles)	Compound formed: dUMP (mμmoles)	Compound formed: Deoxyuridine (mμmoles)
None		3. 06	7. 07	2. 36
dTMP	0. 50	3. 53	3. 11	4. 61
dTTP	0. 24	3. 23	0	7. 92
dCTP	0. 32	0	6. 88	1. 99

*For details on substrate concentration and assay procedure, *see (19)*.

Studies With Crude and Purified Deoxycytidylate Deaminase

The striking effects of dCTP and of dTTP on deoxycytidylate deaminase were further amplified by the demonstration that the inhibitory effects of dTTP on the enzyme could be completely reversed by dCTP (*25*). In addition, it was found that enzyme activity lost upon storage of crude embryo extracts could be restored by dCTP, as was that lost upon incubation at 37° C, or by treatment with Dowex 1-formate.

The Michaelis plot in text-figure 3 documents the effects of dCTP on fresh and aged embryo extracts, and it is seen that while the activity was increased with fresh extracts the most striking results were obtained with aged preparations, particularly at the lower levels of substrate. Thus, at concentrations of 10 mM dCMP, little or no activation by dCTP was observed, but at the 0.2 mM level the enzyme activity was enhanced threefold with freshly prepared embryo extract and more than 20-fold with aged extracts. The apparent increase in affinity of the deaminase for dCMP, in the presence of low levels of dCTP, and its rather potent inhibition by dTTP are results that bear a striking similarity to those described with numerous bacterial systems, and which have fallen into the category of *feedback control* or *end product inhibition* (*26*). However, in this instance two end products are concerned, each exerting an opposite effect on an enzyme that may direct the flow of pyrimidine deoxyribonu-

TABLE 10.—Effect of thymidine nucleotides on chick embryo deoxycytidylate deaminase

Additions	μmoles	dCMP deaminated (μmoles)
None		0. 234
dTTP	0. 02	0. 043
	0. 10	0. 015
dTDP	0. 02	0. 110
dTMP	0. 10	0. 248
	0. 50	0. 072

Each reaction vessel contained 0.5 μmole dCMP-2-C^{14} (1.03×10^5 cpm/μmole), 50 μmoles Tris buffer (*p*H 8.0), 15 μmoles NaF, 0.1 ml of 4-day chick embryo supernatant from a 50 percent homogenate, nucleotides as indicated. Total volume, 0.5 ml. Incubation time, 10 minutes at 37° C. *See (18)*.

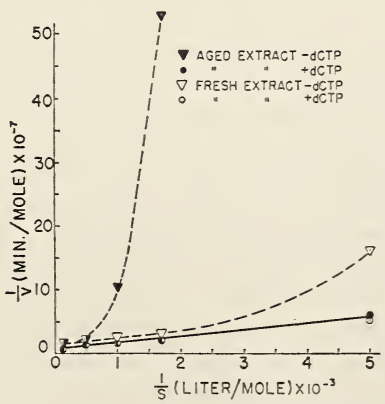
TABLE 11.—Effect of nucleotides on chick embryo deoxycytidylate deaminase*

Additions	μmoles	dCMP deaminated (μmoles)
None		0. 244
dTTP	0. 10	0. 029
dUTP	0. 45	0. 123
dATP	0. 40	0. 227
dGTP	0. 40	0. 226
dCTP	0. 42	0. 332

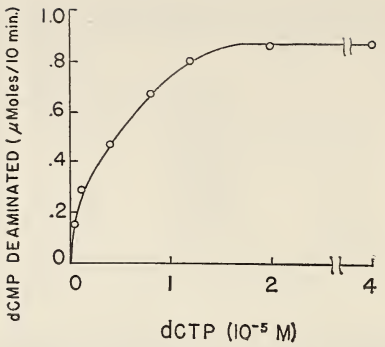
*See table 10 for methods.

cleotides into DNA. It is therefore possible that this enzyme plays an important regulatory role in the synthesis of DNA.

It was desirable to purify the deoxycytidylate deaminase to ascertain more completely the magnitude of the observed effects and possibly the mechanism by which they are brought about. Thus, the enzyme was extensively purified from 6-day chick embryos; the preparative procedures and detailed properties will be described elsewhere. Preliminary studies, some of which have been reported (*25*), reveal that the magnitude of the activation by dCTP is much greater than originally demonstrated in crude extracts, as is the inhibition by dTTP. The purified enzyme has also been inactive in the absence of dCTP at substrate concentrations of dCMP 2 mM or lower. Text-figure 4 reveals the deaminase activity at this substrate concentration as maximal at about 10^{-5} M dCTP. Furthermore, a divalent cation, such as Mg^{++}, Mn^{++}, or Co^{++}, was essential for activity. It should be emphasized though that such requirements were not necessary when the dCMP concentration was at 10 mM. However, the inhibition by dTTP was still demonstrable at this substrate concentration, and was noncompetitive with respect to dCMP ($Ki > 10^{-5}$ M). It is presumed that dCTP and dTTP compete for the same site, but this has been difficult to prove with the assay conditions that are currently employed.



TEXT-FIGURE 3.—Effect of dCTP on deoxycytidylate deaminase in fresh and aged chick embryo extracts. *See* (*25*) for assay procedures.



TEXT-FIGURE 4.—Requirement of purified deoxycytidylate deaminase for dCTP. *See* (*25*) for assay procedure.

Studies with crude extracts previously indicated that dTDP and dCDP exerted effects similar to those exerted by the corresponding triphosphates. However, the diphosphates have essentially no effect on the purified enzyme, and thus again the *end product* nature of observed reactions is emphasized.

The mechanism by which dCTP and dTTP exert their opposing influences upon deoxycytidylate deaminase is purely speculative at present, but it is conceivable that their actions may in some manner alter the conformation or state of aggregation of the enzyme and thus affect its catalytic activity. A number of examples of enzymes that behave in this fashion has recently been reviewed by Monod *et al.* (*27*) under the title of *allosteric* proteins.

CONCLUSIONS

In view of the current interest in mechanisms of metabolic control and regulation, it has become an overwhelming temptation to rationalize the apparent effects of metabolites on enzymes and enzyme systems. We have also succumbed to this temptation, and in the experiments described in this report we attempted to correlate the effects of thymidine on the enhancement of deoxycytidine incorporation in DNA cytosine. But this was not tried until certain properties of the enzymes involved became known and other possible explanations were ruled out. Thus, the potent inhibitory effect of dTTP on both deoxycytidylate deaminase and deoxyuridine kinase presented a plausible explanation for the effectiveness of thymidine in promoting the apparent increased availability of d-cytidine as a precursor of DNA cytosine. The possibility that this effect is brought about by an enhancement of DNA synthesis by thymidine appears to be negated by the experiments presented in table 2. That the enhancement might only be an apparent one, due to an inhibition of the CDP → dCDP conversion by dTTP, would also appear to be discounted by the experiment in table 3. It might be argued that the incorporation

of cytidine into DNA cytosine should also be enhanced by thymidine, but considering that the deoxyribose transformation (text-fig. 1) occurs beyond the site at which dTTP acts seems unlikely and is not supported by the data (table 3).

In view of these considerations, the effective increase in d-cytidine (or dCMP) incorporation into DNA cytosine appears to be related to an inhibition in the synthesis of thymidine derivatives from d-cytidine (which is in essence a sparing effect) and is reflected in an accumulation of d-cytidine, when dCMP-2-C^{14} is employed as a precursor (table 1). These results suggest a block in the d-cytidine→dCMP→dUMP→d-uridine pathway. Associated with this effect is a decrease in the total radioactivity in the d-uridine fraction, in addition to a decrease in the specific activity as the thymidine concentration is raised. Recent experiments revealed that the decrease in the specific activity of d-uridine may be due to an increase in the total d-uridine, which could result from a block in the reversal stage of the last site in the UDP→dUDP→dUMP→d-uridine pathway. An accumulation of a low level of dTTP, above that normally present, could account for the increase in the d-cytidine and d-uridine that is encountered.

One disconcerting aspect in these proposals is the finding that the enhancement effect by thymidine decreases with increasing concentrations of this nucleoside (text-fig. 2). It may be that beyond a certain concentration thymidine becomes inhibitory to other sites subject to inhibition by dTTP, such as the ribose-deoxyribose (*28*) transformation, and results in an inhibition of DNA synthesis. However, this possibility will have to be examined further.

While the speculations presented regarding the data are in accord with the obtained results, they are by no means inviolate and further studies will be necessary to insure the validity of the proposals.

SUMMARY

The mechanism of an apparent sparing effect of thymidine on the utilization of deoxycytidine was examined in chick embryo. It was found that thymidine triphosphate is an effective inhibitor of two enzymes involved in the metabolism of deoxycytidine derivatives, deoxyuridine kinase and deoxycytidylate deaminase. If the addition of thymidine to chick embryo mince enlarges the pool size of thymidine triphosphate, an inhibition of these enzymes may result, which could provide an explanation for the observed enhancement of incorporation of deoxcytidine 5′-monophosphate-2-C^{14} into the cytosine of deoxyribonucleic acid. The possibility that this observed increase may be concerned with an elevation in deoxyribonucleic acid synthesis or an alteration in the pool size of the deoxycytidine nucleotides, due to an inhibition of the ribose-deoxyribose transformation by thymidine triphosphate, seems to be ruled out.

Some properties of crude and purified deoxycytidylate deaminase were discussed, in particular the feedback control of its activity by two opposing nucleotides, deoxycytidine triphosphate and thymidine triphosphate, and the implications this may have in the regulation of pyrimidine deoxynucleotide metabolism.

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MESSENGER RIBONUCLEIC ACID AND PROTEIN SYNTHESIS IN RAT LIVER [1, 2, 3]

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ALTHOUGH less than 3 years have elapsed since the concept of messenger ribonucleic acid (mRNA) (*1*) was formulated, it is universally accepted that this nucleic acid fraction is the template for protein synthesis in bacterial cells. Surely, a similar relationship exists in mammalian cells, but unequivocal evidence has yet to be presented. During recent months we have sought to demonstrate mRNA in rat liver and to define its role in protein synthesis. The following observations, made in our laboratory, are pertinent:

1) In liver cytoplasm there are ribonucleoprotein particles, some of which are capable of synthesizing protein *in vitro* in the presence of, but but not in the absence of, added template.

2) In cytoplasm there is an RNA fraction that rapidly takes up and rapidly loses radioactivity following the administration *in vivo* of an isotopic RNA precursor.

3) In cytoplasm there is an RNA that stimulates amino acid incorporation in an *in vitro* protein-synthesizing system, and its distribution in sucrose gradients corresponds in part to the distribution of rapidly labeled RNA.

4) In rat liver nuclei there is a much more heterogeneous RNA fraction that takes up and loses radioactivity even more rapidly than the early labeled cytoplasmic component.

5) In nuclei there is also a heterogeneous RNA fraction with a high degree of stimulatory activity in an *in vitro* protein-synthesizing system. The distribution of this stimulatory activity resembles that of rapidly labeled RNA.

These observations, taken together, provide strong evidence for the presence in rat liver of mRNA.

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[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27–30, 1963.

[2] Aided by grants from the American Cancer Society, Inc., the Leukemia Society, and the U.S. Public Health Service.

[3] All text-figures are reprinted by permission of the *Journal of Molecular Biology*.

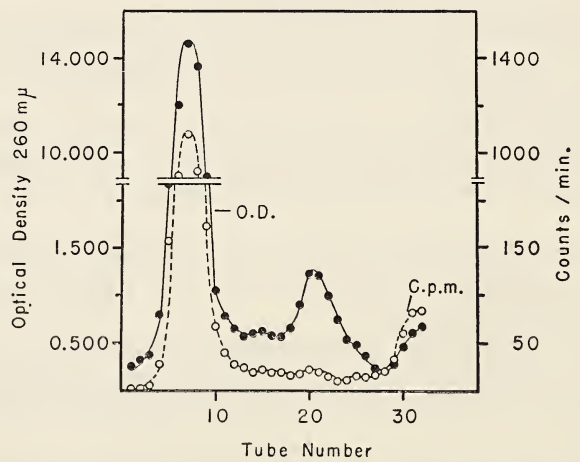
METHODS

Most studies were carried out with material obtained from the livers of male Wistar rats weighing 250 g by procedures described in detail elsewhere (*2*, *3*). The capacity of the purified RNA fractions to stimulate amino acid incorporation into protein was measured with the S30 fraction of Nirenberg and Matthaei (*4*), and these experiments will be presented in more complete form elsewhere (*5*).

RESULTS AND DISCUSSION

Sites of Protein Synthesis in Rat Liver Cytoplasm

It has long been known that proteins are assembled on ribonucleoprotein particles (ribosomes) in rat liver cytoplasm (*6*). Siekevitz and Palade (*7*) have shown that in the pancreas the particles that synthesize digestive enzymes are primarily those attached to a lipoprotein membrane, the endoplasmic reticulum. We have obtained evidence that a similar phenomenon prevails in rat liver in that the particles most active in protein synthesis are those associated with the membrane. Text-figure 1 gives the results of a sucrose gradient centrifugation study of the microsomal fraction (the term applied to the material sedimenting after 1 hour of centrifugation at 105,000 × *g* of the postmitochondrial supernatant



Text-figure 1.—Sucrose gradient analysis of the microsomal fraction of rat liver after *in vivo* labeling with arginine-C^{14}. Twenty μc (2 μmoles) of arginine-C^{14} was injected intraperitoneally into a 24-hour-fasted, 160 g rat 20 minutes before it was killed. The liver microsomal fraction was isolated and subjected to sucrose density gradient centrifugation for 2½ hours and analyzed as described elsewhere (*3*). Tube #1 is from the bottom of the gradient and tube #32 is from the top. The 20 to 50 percent sucrose interface is located at about tube 7. Optical density at 260 mμ (to localize nucleic acid) is indicated by ● and the trichloroacetic acid-precipitable radioactivity by ○.

fraction) from the liver of a rat that received intraperitoneal injections of arginine-C^{14} 20 minutes before it was killed. The pattern of optical density indicated that ribonucleoprotein particles were found primarily at the 20 to 50 percent sucrose interface (tube 7), and that a much smaller number sedimented more slowly, with a peak in tube 21. Evidence has been presented elsewhere (*3*) that the material in and about tube 7 had the characteristics of ribonucleoprotein particles bound to endoplasmic reticulum, while that in tube 21 represented particles free of membrane with the characteristic 80 *S* sedimentation of free liver ribosomes (*8*). The distribution of radioactivity in this gradient demonstrates that the membrane-bound particles incorporated a large quantity of the administered amino acid, while the free particles were relatively inactive in this respect. This indicates that the membrane-bound particles of liver incorporate amino acids more actively than do the free.

The demonstration of this population of free ribosomes was followed by an inquiry into the basis for their relative inactivity with respect to protein synthesis. Specifically, were these particles capable of synthesizing protein? If so, what was required to stimulate them to do so? To answer these questions, membrane-bound and membrane-free particles were isolated by the sucrose gradient centrifugation technique from the livers of rats which had not previously received a radioactive amino acid injection. These particles were then tested separately for their *in vitro* capacity to incorporate a radioactive amino acid into protein. As is evident from columns A and C in table 1, the bound ribosome fraction incorporated phenylalanine-C^{14} much more effectively than did the free. However, in the presence of a synthetic "messenger" polynucleotide, polyuridylic acid, which is known to stimulate phenylalanine incorporation into protein (*4*), the free particles were even more active than the bound (columns B and D). Thus, the free particles were equal to the task of protein synthesis but required instructions in order to do so. Similar observations have been reported by Maxwell (*9*) and by Weinstein and Schechter (*10*).

TABLE 1.—Phenylalanine-C^{14} incorporation *in vitro* by liver particles separated by sucrose density gradient centrifugation*

Fraction	Total radioactivity (cpm)		Specific activity (cpm/μg RNA)	
	A − Poly-uridylic acid	B + Poly-uridylic acid	C − Poly-uridylic acid	D + Poly-uridylic acid
Bound ribosome	77. 0	249	1. 9	6. 1
Free ribosome	7. 6	217	0. 4	11. 0

*The fractions were obtained by pooling tubes from sucrose gradients of the liver microsomal preparations from 10 rats which had not received injections of radioactive material. The gradients were similar to those depicted in text-figure 1, except for the absence of radioactivity. The bound ribosome fraction was taken from material corresponding to tubes 4 to 9, and the free ribosome fraction from tubes 18 to 24 in text-figure 1. These fractions were pooled and centrifuged at 100,000 × *g* for 3 hours to sediment the particles. These particles were then assayed for their capacity to incorporate phenylalanine-C^{14} into protein in the presence and the absence of polyuridylic acid, as described elsewhere (*3*).

The presence of particles requiring template for function implies the existence in liver cytoplasm of mRNA. In our search for mRNA we anticipated that such a fraction might manifest certain characteristics of bacterial messenger, including rapidity of uptake of radioactivity after exposure of the cell to an isotopic RNA precursor (*11*), and a capacity to stimulate amino acid incorporation in an *in vitro* protein-synthesizing system (*4*). These criteria were used to identify a messenger-like fraction in rat liver cytoplasm.

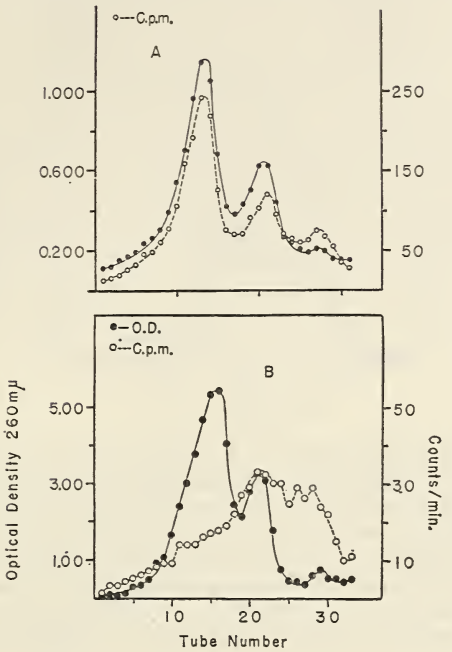
Messenger RNA in Liver Cytoplasm

Labeling of microsomal RNA.—RNA was isolated with sodium dodecyl sulfate and phenol from the microsomal fraction of livers of rats that had received injections of orotic acid-C^{14} 30 minutes or 24 hours previously. Examination by sucrose density gradient centrifugation revealed a striking difference in the RNA obtained under these two conditions (text-fig. 2). The optical density pattern in both circumstances was similar, with 2 principal peaks in each corresponding to the 28 *S* and the 18 *S* RNA of liver ribosomes (*12*). The RNA obtained 24 hours after the administration of the radioactive precursor (text-fig. 2A) showed a pattern of radioactivity that coincided with that of optical density. In sharp contrast was the appearance after 30 minutes of labeling (text-fig. 2B). Here, there was a peak of radioactivity, approximating 6 to 20 *S*. Total radioactivity in this 6 to 20 *S* fraction was not so great as that in transfer RNA after purification of total cytoplasmic RNA. The 6 to 20 *S* fraction was previously described by Munro and Korner (*13*).

Stimulatory capacity of microsomal RNA.—Microsomes from 10 rat livers were combined, and the RNA was isolated with sodium dodecyl sulfate and phenol and subjected to sucrose density gradient centrifugation. RNA fractions were separated according to sedimentation pattern, precipitated with alcohol, dried, and then tested for their capacity to stimulate amino acid incorporation into protein in the *in vitro* system derived from *Escherichia coli* (*4*). Text-figure 3 depicts the sedimentation of the RNA fractions pooled and labeled 1 through 6 and the capacity of these fractions to stimulate amino acid incorporation into protein. Stimulatory activity was found throughout the gradient, with the highest level per tube in fraction 4. Its sedimentation corresponded only in part to that of the material most rapidly labeled after administration of a radioactive precursor of RNA (text-fig. 2B).

Nuclear Messenger RNA

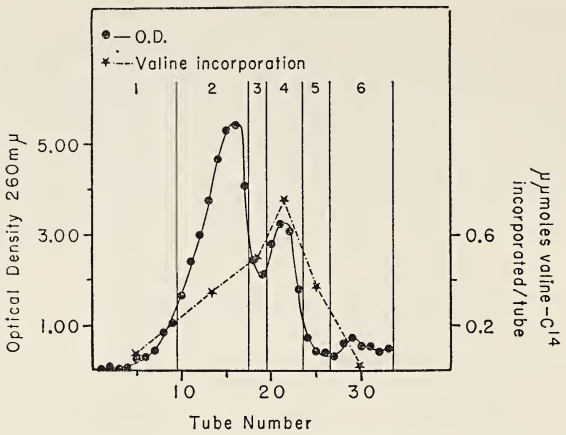
Clearly, the synthesis of cellular mRNA requires the presence of DNA as template. [The messenger of certain RNA viruses is believed to be synthesized with viral RNA as template (*14*).] Thus, it might be anticipated that mRNA is synthesized in the nucleus. RNA has been purified from rat liver nuclei and has been examined for its pattern of uptake



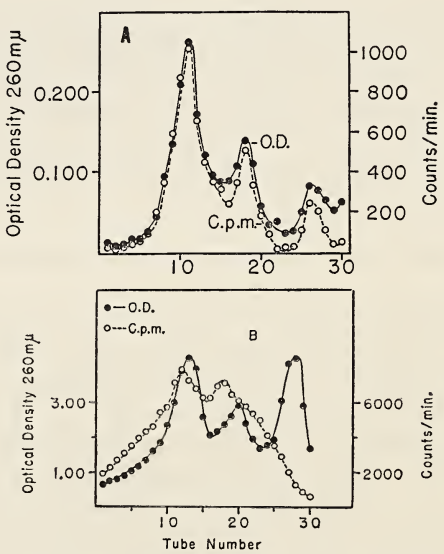
TEXT-FIGURE 2.—Sedimentation pattern of labeled microsomal RNA. Thirty minutes (B) or 24 hours (A) after the intraperitoneal administration of orotic acid-C^{14}, 250 g rats were killed, the livers removed, and the microsomal fractions isolated. The preparation of RNA by extraction with sodium dodecyl sulfate and phenol and sucrose gradient analyses were carried out as described elsewhere (*2*). Optical density at 260 mμ (to identify RNA) is indicated by ●, and the trichloroacetic acid-precipitable radioactivity by ○. The rats represented in experiment A received injections of 1.5 μc per 100 g of body weight, and those in B received 10.

of radioactivity and for its stimulatory activity with respect to *in vitro* protein synthesis.

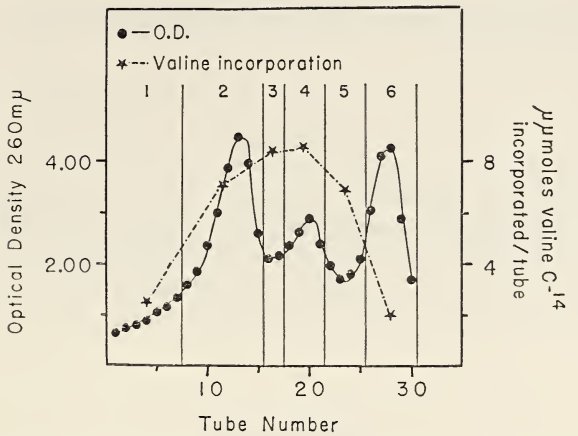
Nuclear RNA from the livers of animals which had received injections of orotic acid-C^{14} 30 minutes or 24 hours before they were killed was analyzed by sucrose density centrifugation (text-fig. 4). The principal peaks of optical density corresponded to the 28 *S* and 18 *S* fractions observed in the cytoplasm, and at 24 hours the patterns of radioactivity and optical density were superimposable (text-fig. 4A). The early labeled RNA in the nucleus, however, was more abundant than that observed in cytoplasm, it was much more heterogeneous, and its average sedimentation coefficient was much greater. RNA isolated from the nuclei of several rat livers was fractionated according to sedimentation pattern by the sucrose gradient procedure. The addition of these fractions to the *in vitro* protein-synthesizing system from *E. coli* revealed marked differences from the results observed with cytoplasmic RNA. Not only did the RNA of the nuclear fractions have a higher proportion of material capable of stimulating amino acid incorporation into protein,



TEXT-FIGURE 3.—Sedimentation pattern of microsomal RNA and capacity of fractionated RNA to stimulate amino acid incorporation into protein *in vitro*. Microsomal RNA was prepared and subjected to sucrose gradient centrifugation as described in text-figure 2. The tubes were pooled according to the groupings represented by the vertical lines into fractions 1 through 6. RNA from each of these pooled fractions was precipitated with alcohol, dried, and assayed for its capacity to stimulate amino acid incorporation into protein as described elsewhere (*5*). *Star-shaped points* represent μμmoles of valine-C^{14} incorporated per tube in each fraction (average).



TEXT-FIGURE 4.—Sedimentation pattern of labeled nuclear RNA. Twenty-four hours (A) and 30 minutes (B) after the intraperitoneal administration of orotic acid-C^{14}, rats were killed and their livers removed and fractionated. Nuclear RNA was prepared by extraction with sodium dodecyl sulfate and phenol as described elsewhere (*2*). Sucrose gradient centrifugation analyses were carried out as described (*2*). The rats in A received injections of 1.5 μc of orotic acid-C^{14} per 100 g of body weight, and those in B received 10. Numbers representing the optical density scale in A should be multiplied by a factor of 10.



TEXT-FIGURE 5.—Sedimentation pattern of nuclear RNA and capacity of fractionated nuclear RNA to stimulate amino acid incorporation into protein *in vitro*. Nuclear RNA was prepared and subjected to sucrose density centrifugation as described in text-figure 4. Tubes were pooled in groups, indicated by *vertical lines*, and labeled 1–6. RNA from each of the pooled fractions was precipitated with alcohol, dried, and assayed in the *in vitro* amino acid incorporating system from *E. coli* described by Nirenberg and Matthaei (*4*). *Star-shaped points* indicate μμmoles of valine-C^{14} incorporated into protein per tube in each fraction (average).

but the stimulatory activity was much more heterogeneous in sedimentation (text-fig. 5).

The relationship of nuclear mRNA to that found in the cytoplasm is not clear. It is assumed that mRNA is synthesized in the nucleus and then transferred to the cytoplasm, though evidence for such a phenomenon is still lacking. The heterogeneity of the nuclear material, both with respect to its pattern of early labeling and to its capacity to stimulate amino acid uptake, is in contrast to the greater localization of these characteristics in microsomal RNA to material of slower sedimentation. The substantial quantities of nucleases known to exist in liver might well result in the transformation of the large and heterogeneous fraction noted in our nuclear preparations to the more slowly sedimenting fraction observed in the cytoplasm. A much larger concentration of mRNA in nucleus as compared with cytoplasm is suggested by previous studies of its capacity to stimulate amino acid incorporation (*15*), as well as our own. This difference may explain the wide discrepancy in base composition of nuclear and cytoplasmic RNA (*16*). The presence of more of a metabolically active RNA in nucleus is further suggested by the demonstration (*17*) in nucleus, but not in cytoplasm, of an enzymatic activity with properties similar to those of enzymes believed to degrade mRNA in bacterial cells (*18*, *19*).

The demonstration in liver of ribonucleoprotein particles that require template to synthesize protein and of a purified fraction with the characteristics of template RNA supports the applicability of the mRNA concept to rat liver. The basis for the quantitative differences in RNA of nucleus

and cytoplasm and the possibility of qualitative differences are subjects of current investigation.

SUMMARY

Ribonucleoprotein particles associated with endoplasmic reticulum in rat liver cytoplasm incorporate amino acids into protein more actively than do particles free of membrane. The free particles, however, are equally active *in vitro* in the presence of synthetic mRNA. Evidence has been presented for the existence of mRNA in rat liver cytoplasm and nuclei.

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REGULATION OF PROTEIN SYNTHESIS IN THE EMBRYOGENESIS OF THE SEA URCHIN[1,2,3]

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A RAPID increase in protein synthesis occurs after fertilization of the sea urchin egg (*1*). To determine the reason for this change and to understand the mechanisms for the regulation of protein synthesis through embryonic development, the enzymic and nucleic acid components involved were studied by two distinct approaches: 1) Cell-free ribosomal preparations from eggs and embryos were examined for their ability to execute the two sequential sets of reactions necessary for protein synthesis; namely, the production of specific amino acyl soluble RNA's (*2*) and the assemblage of the amino acids into polypeptide chains specified by messenger RNA. 2) The major components of RNA of the sea urchin egg were characterized by sedimentation analysis and their fate in the course of development was examined together with the nature of the RNA newly synthesized at various embryonic stages.

METHODS AND MATERIALS

Eggs and embryos.—For studies on polypeptide synthesis, the species *Arbacia punctulata* and *Lytechinus pictus* were used. For RNA synthesis *Strongylocentrotus purpuratus* was studied. Eggs were fertilized and allowed to develop (*3*) in artificial sea water at 20° C in the presence of penicillin and streptomycin (30 and 50 mg/ml, respectively).

Assay of polypeptide synthesis.—Polypeptide synthesis was measured after incorporation of labeled amino acids (either phenylalanine or leucine) by a homogenate fraction, prepared as follows: At various developmental stages eggs were packed by light centrifugation in homogenizing vessels, and an equal volume of homogenization medium (*1*), containing 0.006 M mercaptoethanol was added. After gentle homogenization at 0 to 5° C, the homogenate was centrifuged for 10 minutes at 12,000 × *g*. The supernatant fluid (approximately 1 ml) was passed through a column

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[3] *Abbreviations used:* polyU, polyuridylic acid; polyUG, polyuridylic-guanylic acid; PEP, phosphoenolpyruvate; GTP, guanosine triphosphate; RNA_p, pulse RNA; RNA_c, chase RNA.

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(1.2 × 10 cm) of Sephadex-25 equilibrated with incubation medium, which was the same as the homogenization medium except that sucrose was omitted. The front of the effluent solution, which had been effectively dialyzed against incubation medium, was called the S-12 fraction, and was adjusted to contain 0.50 mg per ml RNA and approximately 5 mg per ml protein (*4*, *5*).

Incubations were performed with 0.2 ml of S-12 fraction at 30° C for 45 or 60 minutes. The rate of incorporation of phenylalanine was constant up to 1 hour. The reactions were stopped with addition of trichloroacetic acid (to 5%). Samples were heated at 90° C for 20 minutes, and the precipitates were washed and plated on glass-fiber discs. Radioactivity was measured in a gas-flow counter with a Micromil window and a counting efficiency of approximately 30 percent. Samples were counted at infinite thinness. All assays were in duplicate.

Incorporation in RNA.—At various developmental stages, 1 cc of eggs or embryos was settled by light centrifugation and suspended to 5 cc with sea water for 10 or 20 minutes, during which time they were exposed to a concentration of either 3.5 or 7 μM uridine-H^3 (2.77 mc/μmole; New England Nuclear Corporation). At the end of this pulse period the eggs were suspended in sea water containing unlabeled uridine (5 mM). Half of this suspension was centrifuged quickly. The embryos in the pellet were frozen immediately to −80° C and stored. The other half of the suspension was centrifuged lightly, then resuspended in 100 ml sea water containing 0.5 mM uridine. These continued developing for 4 hours. After this chase period the embryos were centrifuged and their pellet was frozen and stored as described

Extraction of RNA.—For further processing the eggs or embryos were thawed and homogenized in a solution of 0.5 percent dodecyl sulfate, 0.01 M $MgCl_2$, 0.1 M NaCl, and 0.01 M acetate buffer, *p*H 6.0. Samples were immediately submitted to phenol extraction at 60° C (*6*, *7*).

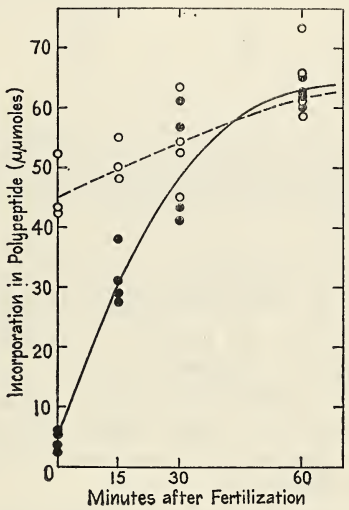
Sedimentation analysis.—Purified RNA in a solution containing 0.01 M $MgCl_2$, 0.1 M NaCl, and 0.01 M acetate buffer, *p*H 6.0, was applied to a 5 to 20 percent linear sucrose gradient (*8*) in the same solution, containing 100 μg per ml bentonite. Samples were centrifuged for 11 hours at 25,000 rpm in the Spinco head SW25.1. Equal fractions were collected. Optical densities at 260 mμ were determined and the radioactivities of aliquots of the fractions in scintillation fluid (*9*) were measured in a scintillation counter. At least duplicate pulse-chase experiments with different batches of eggs were performed for every embryonic stage represented. All pertinent figures have been normalized to contain a total of 10 optical $density_{260}$ units of RNA per gradient.

The sedimentation constants of the various RNA peaks were estimated by addition to the sucrose gradients of a trace of P^{32}-labeled RNA, prepared from strain L cell fibroblasts (gift of R. Perry), containing components of known sedimentation constants (*10*). With the 18S component of the P^{32}-marker RNA as a standard, the approximate S values of the peaks were derived by the method of Martin and Ames (*11*).

RESULTS AND CONCLUSIONS

Polypeptide Synthesis

The apparent check on protein synthesis in the unfertilized egg may result from limitations on the concentrations of enzymatic components responsible either for the production of specific amino acyl sRNA's or the assemblage of polypeptides. Although the endogenous protein synthesis measured by phenylalanine incorporation with the S-12 fraction of unfertilized eggs is very low, a 200-fold increase in the incorporation of phenylalanine in polypeptide can be obtained by the addition of polyU to the system (text-fig. 1 and table 1). When polyUG is used as template RNA, an 11-fold stimulation of leucine incorporation is seen (*see* table 3).



TEXT-FIGURE 1.—Polyphenylalanine and protein synthesis by S-12 fraction of *Arbacia punctulata* before and after fertilization. Incorporation in protein is ●; in polyphenylalanine is O. The reaction mixture contained the following in μmoles per ml: 50 Tris buffer, *p*H 7.8; 10 $MgCl_2$; 240 KCl; 6 mercaptoethanol; 1 ATP; 5 PEP; 0.06 GTP; and 7 mμmole per ml L-phenylalanine-C^{14} (150,000 counts/min). The total volume of 0.85 ml also contained 20 μg PEP kinase; 3 mg S-12 protein; 0.5 mg polyU or a mixture of 20 L-amino acids minus phenylalanine each 22 μmole per ml. Incubations were at 30° C for 60 minutes. Incorporation of phenylalanine in protein (in absence of polyU) has been multiplied by 20.

The rate of protein synthesis in the S-12 fractions of *A. punctulata* increased 14-fold during the first 60 minutes after fertilization; at the same time polyphenylalanine synthesis increased less than 1.5-fold (text-fig. 1).

The maximal velocities (Vmax) of polyphenylalanine synthesis were determined in the presence of excess yeast sRNA in S-12 fractions of *L. pictus* prepared at different embryonic stages. Text-figure 2 shows the ratio of polyU concentration to polyphenylalanine concentration

TABLE 1.—Incorporation in polypeptide by S-12 fractions of various embryonic stages of *Lytechinus pictus**

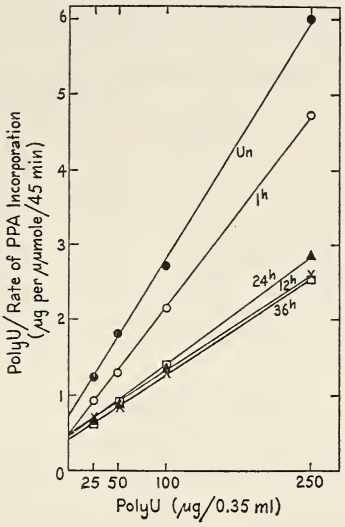
Polypeptide	Incorporation in polypeptide ($\mu\mu$moles/100 μg S-12 RNA)			
	Unfertilized	Cleavage	Blastula	Gastrula
		Batch No. 1		
Protein	5	24	50	
PPA	51	65	75	
		Batch No. 2		
Protein	5	16	63	58
PPA	49	59	106	96
		Batch No. 3		
Protein	2	9		40
PPA	21	51		82
Average ratio PPA/protein	10	3. 6	1. 6	1. 8

* S-12 fractions from 3 batches of eggs were assayed for PPA (polyphenylalanine) synthesis according to conditions in text-figure 2; except the yeast sRNA concentration and the polyU were each 0.5 mg per ml. For protein synthesis a mixture of 20 L-amino acids minus the labeled amino acids was present at 0.22 μmole per ml. Protein synthesis for batch 2 was measured by incorporation of L-leucine-C^{14}, 1 mμmole per ml. Phenylalanine incorporation in protein was multiplied by 20, and leucine incorporation by 8.

plotted against polyU concentration, in accordance with the Lineweaver-Burk modification of the Michaelis-Menten relation (*12*). The calculated Vmax increased from 50 $\mu\mu$moles of phenylalanine incorporated in 45 minutes in the unfertilized egg to 60 in the 1-hour zygote, to approximately 100 in the 12-hour blastula, where it remained constant through the 24- and 36-hour gastrula stages. Thus the capacity of the system for polyphenylalanine synthesis increased with development to a value twice that of the unfertilized egg.

In the development of *L. pictus* to blastulae the rate of protein synthesis increased considerably, but further development to gastrulae took place with much less increase (table 1). In this second phase the ratios of polyphenylalanine to protein synthesis were essentially constant (table 1); that is, they changed at the same rate.

Protein synthesis and the synthesis of polypeptide directed by synthetic messenger polyribonucleotides were studied in the presence of an excess (*3*) of yeast sRNA or in the absence of added yeast sRNA in the S-12 fractions derived at various stages of development. In the presence of an excess of polyU, the concentration of endogenous sRNA in the S-12 fraction of unfertilized eggs of *L. pictus* could support only 9 percent of the polyphenylalanine synthesis that could be achieved in the presence of an excess of yeast sRNA (table 2). In the course of development (table 2) there was a substantial increase in this synthetic capacity determined by the concentration of endogenous sRNA. In the presence of polyUG, the S-12 fraction from eggs of *A. punctulata* allowed 59 percent of the incorporation of leucine in polypeptide attained with added yeast sRNA (table 3). At the blastula stage, the endogenous concentration of



TEXT-FIGURE 2.—Effect of concentration of polyU on polyphenylalanine synthesis by the S-12 fraction of *Lytechinus pictus* at various stages of development: ● unfertilized egg; ○ 1-hour zygote; x 12-hour blastula; ▲ 24-hour gastrula; □ 36-hour gastrula. The reaction mixture was the same as that of text-figure 1, except the concentration of yeast sRNA was 0.7 mg per ml, and polyU was varied as indicated on the abscissa. The ratio of polyU concentration to rate of PPA incorporation is given on the ordinate. The volume of the reaction mixture of 0.35 ml contained 1 mg protein. L-phenylalanine-C^{14} was 3.5 μM.

sRNA was completely adequate to support the requirements of leucine incorporation in the presence of polyUG. PolyUG presents much less demand on the supply of leucyl sRNA than polyU on the supply of phenylalanyl sRNA. In both cases the supply of endogenous sRNA appears to increase with development.

In both species the addition of yeast sRNA had little effect on the incorporation of leucine or phenylalanine in protein. Unless yeast sRNA is lacking in transfer RNA's specific for the sea urchin, endogenous levels of sRNA are adequate at all stages for the needs of protein synthesis.

TABLE 2.—Dependency on added yeast sRNA for protein and polyphenylalanine synthesis at different stages of development in *Lytechinus pictus**

Stage	Leucine incorporation in protein (μμmoles/100 μg S-12 RNA)			Phenylalanine incorporation in polyphenylalanine (μμmoles/100 μg S-12 RNA)		
	−sRNA	+sRNA	Percent capacity	−sRNA	+sRNA	Percent capacity
Unfertilized	0. 56	0. 64	87	3. 9	42	9
1-Hour zygote	1. 83	2. 07	88	4. 3	53	8
12-Hour blastula	5. 48	7. 85	70	14. 7	96	15
24-Hour gastrula	6. 74	7. 25	93	22. 2	90	25

*Conditions were the same as those for table 1. A mixture of 20 L-amino acids minus leucine, 0.22 μmoles per ml; L-leucine-C^{14}, 1 mμmole per ml; polyU, 0.7 mg per ml in incubations with L-phenylalanine-C^{14}. Yeast sRNA, 0.7 mg per ml.

TABLE 3.—Dependency on added yeast sRNA for incorporation of leucine in polypeptide in presence and absence of polyUG in *Arbacia punctulata**

Stage	Leucine incorporation in polypeptide ($\mu\mu$moles/100 μg S-12 RNA)					
	−PolyUG			+PolyUG		
	−sRNA	+sRNA	Percent capacity	−sRNA	+sRNA	Percent capacity
Unfertilized	0. 41	0. 46	91	2. 93	5. 00	59
1-Hour zygote	2. 43	2. 58	94	4. 28	6. 58	65
12-Hour blastula, late	3. 93	3. 85	102	6. 18	6. 28	98

*Conditions were the same as those of table 2. PolyUG (5 : 1), 0.6 mg per ml.

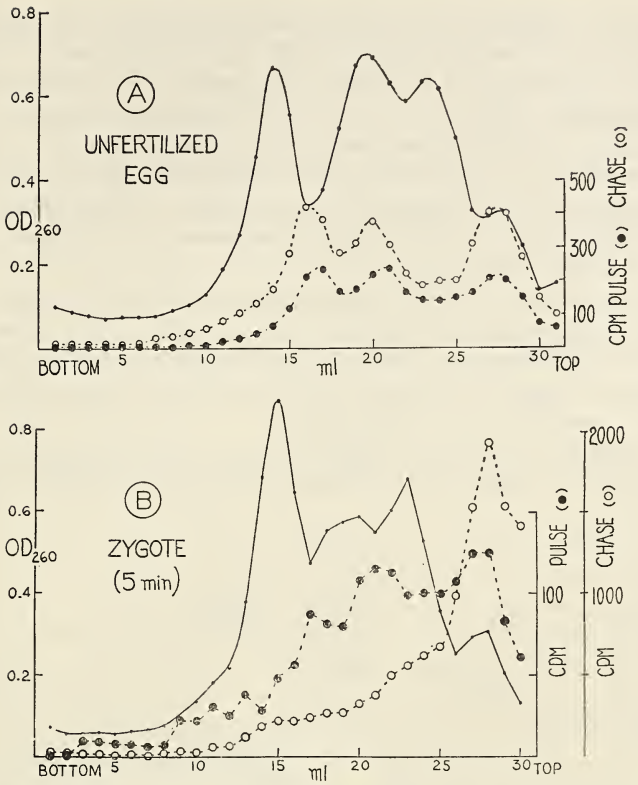
RNA, Old and New

Chemical measurements indicate that there is practically no change in the amount of RNA present through early development (*13*, *14*). Nevertheless, incorporation of various radioactive precursors into the RNA of the sea urchin embryo has been detected (*15–18*). If this incorporation does not entail a net increase, a substantial net synthesis of any one RNA compartment would have to come at the expense of another. An increase with development in the concentration of one such compartment, that of transfer RNA, as deduced, will be corroborated by the following data. This study was undertaken to examine more closely the role of RNA, that which is stored in the egg as well as that which is synthesized allowing the issuance of new genetic information.

Pulse and Chase RNA

The sedimentation pattern of incorporation following a short exposure (pulse) to radioactive precursor has been compared to that at the end of a subsequent incubation in the presence of unlabeled precursor (chase). The portion of radioactive RNA of the pulse destined for stable components will be found associated with them following the chase. On the other hand, any rapidly degraded RNA will only be detectable after a pulse and not at the end of a chase. The course of embryogenesis of the sea urchin, it will be demonstrated, is marked by striking changes in the character of pulse and chase RNA (RNA_p and RNA_c, respectively).

Unfertilized eggs.—Nucleosides are utilized to a lesser degree before than after fertilization (*19*); nevertheless, their incorporation into the RNA of the unfertilized egg is detectable. The characteristics of this incorporation were studied by sedimentation analysis of RNA from eggs that had been incubated with H^3-uridine for a 20-minute pulse and those that had been incubated in unlabeled uridine for a 4-hour chase, following the 20-minute pulse. After the pulse, radioactivity was distributed through the gradient into peaks of approximately 4, 17, and 22S (text-fig. 3A). Following the chase, the same distribution persisted, but with a slight increase in specific activity. In both cases the 4S RNA was the



TEXT-FIGURE 3.—Sedimentation diagrams of RNA from (A) unfertilized and (B) just-fertilized eggs. Eggs were pulsed for 20 minutes with H^3-uridine (7 μM) and chased for 4 hours in the presence of 0.5 mM unlabeled uridine. *Solid curve* represents absorbancy at 260 mμ. *Dotted lines* represent radioactivities of pulse (●) and chase (○).

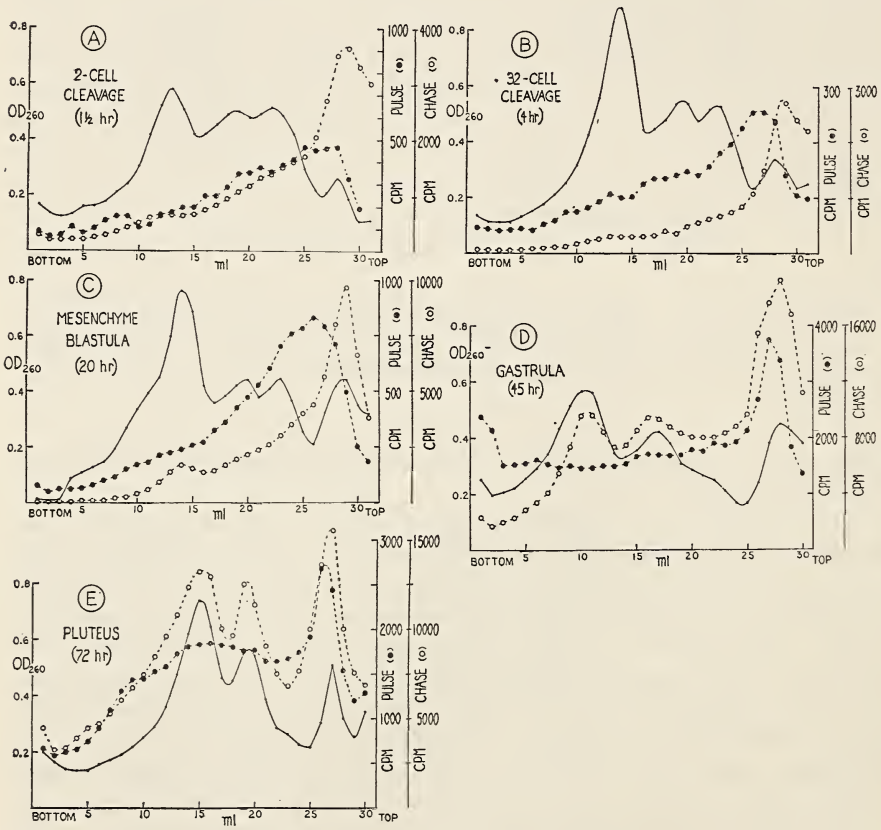
only major component labeled. The 17 and 22S components appeared relatively stable.

Just-fertilized egg.—Exposure of eggs 5 minutes after fertilization to a 20-minute pulse of H^3-uridine resulted in a pattern of incorporation similar to that of the unfertilized egg (text-fig. 3B). Any expected difference between the unfertilized and fertilized egg is manifested only quantitatively, since the latter has an appreciably greater specific activity. In contrast to the behavior of the unfertilized egg, a 4-hour chase results in the incorporation into all the major RNA components, predominantly into the 4S RNA (text-fig. 3B). The increase in specific activity during the chase is too great to decide whether the pulse RNA peaks at 17 and 22S have disappeared or have been obscured by the pattern of the chase.

Pregastrula stages.—Embryos at various stages were exposed to H^3-uridine for a pulse of 10 minutes. Half of these were further incubated in the presence of unlabeled uridine for a chase of 4 hours. At the 8- to 16-cell cleavage stage, the pulse radioactivity of the extracted RNA

was spread throughout the sucrose density gradient but with a peak displayed at approximately 10S (text-fig. 4B). Incorporation during the chase was mainly into the 4S RNA, with appreciable labeling of the 13S RNA and smaller activity in the 28 and 18S regions. The same pattern was displayed up to the 24-hour mesenchyme blastula stage (text-fig. 4C). During development to this stage, the pulse-labeled peak at 10S became progressively broadened toward the heavy region of the gradient and the degree of utilization of labeled uridine increased. Text-figure 4A represents an incubation following the first division (1½ hours). The character of the RNA_D is intermediate between that of the just-fertilized egg and those in the cleavage and blastula stages; that is, it is apparently a composite containing the 17 and 22S peak as well as the 10S peak.

Postgastrula stages.—A pronounced change in the characteristics of the pulse RNA occurred after gastrulation. In the pulse of the 45-hour gastrula (text-fig. 4D) the 10S region no longer predominated in amount of incorporation. Instead, the radioactivity was dispersed throughout



TEXT-FIGURE 4.—Sedimentation diagrams of RNA from embryos at various stages: Embryos were pulsed for 10 minutes with H^3-uridine (3.5 μM) and chased 4 hours in the presence of 0.5 mM unlabeled uridine. Representations are the same as for text-figure 3: pulse (●) and chase (○).

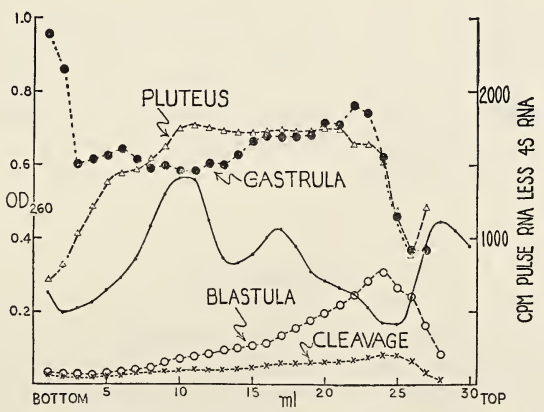
the gradient with substantial amounts present in regions of greater than 30S. The corresponding chase RNA was also different. A large amount of incorporation was found for the first time in the 28 and 18S RNA's. The optical absorbancy of the 13S RNA described only a shoulder instead of a large peak, and little activity was associated with it. In the prism (60 hours) and in the 72-hour pluteus (text-fig. 4E) the pattern of incorporation of pulse RNA was similar to that seen in the gastrula. After chasing, a large amount of activity was found in the major RNA components.

Approximately 6 percent of the total RNA of the egg is 4S. This value rises by a factor of 2 and 3 when the blastula and gastrula stages, respectively, are reached (text-fig. 4). This increase agrees with that seen for transfer RNA in the two other species studied.

Relative Amounts of Synthesis of Various RNA's During Development

The 4S and non-4S RNA can be viewed separately by graphically subtracting the activity (the product of the specific activity and total optical density units) of the 4S RNA from that of the total RNA. The resultant non-4S RNA's of the pulses of various stages are represented on a single scale in text-figure 5 against the optical density pattern of the gastrula stage. Clearly, with development the degree of labeling increases as markedly as the pattern of sedimentation behavior changes. The shift to the heavier region of the gradient is coincident with the pronounced rise in incorporation in ribosomal RNA at gastrulation.

The rapidly synthesized non-4S RNA consists of a component (*m*) that is rapidly degraded, concordant with its function as messenger, and a component (*pr*) that is precursor to stable or ribosomal RNA (*s*) (7). The fraction, $pr/(m + pr)$, of the rapidly synthesized RNA ($m + pr$) converted to *s* can be estimated for an interval which is long enough for



TEXT-FIGURE 5.—A comparison of the sedimentation characteristics of non-4S pulse RNA at various embryonic stages obtained by graphically subtracting the radioactivity of 4S RNA from the pulse RNA of text-figure 4.

essentially all of *pr* to be converted to *s*. An adequate chase represents such an interval. Thus, if the first RNA products (*4S RNA* + *m* + *pr*) in a given interval are derived from the same precursor pool, *P*, then

1) $P_c/P_p = (m + pr)_c/(m + pr)_p = 4S\ RNA_c/4S\ RNA_p$ or $(m + pr)_c = (m + pr)_p\ (4S\ RNA_c/4S\ RNA_p)$.

2) If $s = pr$, then

$pr/(m + pr)_c = s/(m + pr)_p\ (4S\ RNA_c/4S\ RNA_p) =$ non-$4S\ RNA_c$/(non-$4S\ RNA_p$) $(4S\ RNA_c/4S\ RNA_p)$.

Calculations of the fraction of the total radioactivity (cpm) of the rapidly synthesized non-4S RNA, as represented in text-figure 5, converted to stable non-4S RNA yield the values given in table 4. In the pregastrula stages this fraction was approximately 0.33. But only half of this was represented by the known ribosomal 28 and 18S RNA's; the other half was 13S RNA. The postgastrula stages retained much more of the rapidly synthesized RNA in the ribosomal constituents, about 0.52 in the 45-hour gastrula and 0.59 in the 72-hour pluteus.

TABLE 4.—Interrelationships involving 4S and non-4S RNA of *Strongylocentrotus purpuratus*

Stage	Hour	Ratio of incorporation (range)	
		$s/(m + pr)$*	s/4S RNA_c
Cleavage	1–10	0. 31 (0. 30–0. 33)	0. 54 (0. 53–0. 56)
Blastula	20–28	0. 34 (0. 32–0. 38)	0. 87 (0. 65–1. 10)
Gastrula	45–49	0. 52	1. 14
Pluteus	72–76	0. 59 (0. 55–0. 62)	2. 20 (1. 9–2. 50)

*Fraction of rapidly synthesized non-4S RNA ($m + pr$) converted to stable or ribosomal RNA (s = non-*4S RNA*$_c$).

While the ratios $s/(m + pr)$ and thus s/m apparently increase in the course of development, the ratio s/4S RNA_c also increases (table 4). This ratio rises from a value of 0.5 in the early cleavage stages (again including 13S RNA) to 2.2 in the 72-hour pluteus. Therefore, during development the synthesis of ribosomal RNA increases relative to both messenger and transfer RNA.

DISCUSSION

Before and after fertilization.—The incorporation of phenylalanine or leucine into polypeptide is stimulated greatly by the addition of synthetic messenger polyribonucleotides. Since this incorporation is coupled to the formation of phenylalanyl or leucyl sRNA (*20*) through activating and transfer enzymes, the activities of these enzymes apparently considerably exceed the needs of protein synthesis in the egg.

The number of amino acid residues incorporated into protein and into polypeptide, induced by synthetic RNA template, can be compared: Phenylalanine is 5 percent and leucine is 12 percent of the amino acids incorporated in this protein (*3*). The values for their incorporation can be multiplied by 20 and 8, respectively, to represent the amount of incorporated amino acid residues. Less than 10 percent of the polypeptide synthesized on polyUG (5 : 1) template is leucine (*20*). The unfertilized egg can then incorporate tenfold more amino acid residues into polyphenylalanine than into protein, and can synthesize tenfold more polypeptide, directed by polyUG, than protein. If activities tested with synthetic messenger polyribonucleotides are comparable to endogenous protein synthesis, then the polypeptide synthetic capacity of the enzymes and ribosomes of the unfertilized egg exceeds the observed amount of protein synthesis, and cannot be a limiting factor.

Thus the unfertilized egg contains a large reservoir of ribosomes, whose ability to synthesize protein may be limited by a lack of mRNA. The ribosomal capacity for protein synthesis increases after fertilization, apparently because mRNA becomes newly available. A messenger component, if there is any, ought to be demonstrable in rapidly synthesized RNA, since its function as template has been shown to involve a rapid metabolic turnover (*21*). Before and after fertilization in the species *S. purpuratus*, the RNA_p contained two peaks of approximately 17 and 22S. These sedimentation values are also characteristic of bacterial RNA. The extremely high concentration of radioactivity could have served to detect a minute amount of bacterial contamination and yet could have failed to reveal RNA synthesis attributable to the egg itself. If there is synthesis of mRNA following fertilization, it is not apparent from these results.

Developmental patterns of RNA synthesis.—During the early cleavage stages, the nature of the RNA_p experiences a transition (text-fig. 4A) from the pattern of the unfertilized egg to a pattern with a predominant peak of approximately 10S (text-fig. 4B). This may be a transition between the synthesis of messengers patterned by the maternal genome for use before and immediately after fertilization and that of messengers to be involved in the developmental process. This pattern of rapidly synthesized RNA, appearing in all of the pregastrula stages, exhibits a progressive developmental change, whereby the peak broadens toward the heavier region of the gradient. This peak may be a group of mRNA's whose average size increases during this period of development.

The final form taken by RNA_p in this progression occurs in all the postgastrula stages. Its sedimentation pattern is polydisperse with a large proportion of radioactivity of greater than 30S. It is a pattern that has been demonstrated in highly developed growing cells (*7*, *22*). The sharp rise in ribosomal RNA synthesis, seen in chase experiments with gastrulae, coupled with the appearance of substantial radioactivity in RNA_p greater than 30S, agrees with the hypothesis of Scherrer, Latham, and Darnell (*22*) that this part of the RNA_p is precursor to ribosomal

RNA. The calculated proportion of the rapidly synthesized RNA converted to 28 and 18S RNA rises from less than 20 percent in the early cleavage stages to 60 percent in the late pluteus (table 4).

Old and new RNA in the regulation of protein synthesis.—The reservoir of functional ribosomes in the unfertilized egg may largely serve the needs of the embryo in the early stages. A great difference in the increases in the ability of embryonic extracts to synthesize polypeptide where the messenger is in excess (polyphenylalanine synthesis) as compared to where the messenger is apparently limiting (endogenous protein synthesis) is evident in the stages of *L. pictus* before the blastula (table 1). During that period, a greater emphasis may have been placed on the synthesis of mRNA to program the functional ribosomes already present than to construct new ribosomes. Thus endogenous protein synthesis increased more rapidly than polyphenylalanine synthesis. However, a comparison of the blastula and gastrula revealed that further increases were approximately the same for polypeptide synthesis limited and not limited by messenger. Apparently in a second phase of development the emphasis on messenger synthesis was reduced. This succession of periods of relatively high and low proportions of messenger synthesis was demonstrated in the pattern of RNA synthesis of *S. purpuratus*. The ratio of synthesis of labile non-4S RNA to that of stable non-4S RNA (messenger to ribosomal) was greater in the pregastrula than in the postgastrula stages (table 4). Such a demarcation in the nature of the synthesis of RNA may be expected to influence the kinds of regulation of protein synthesis during embryogenesis.

SUMMARY

Control of protein synthesis in the embryogenesis of the sea urchin was studied through examination of 1) the activities of the components of cell-free ribosomal systems, prepared from *Arbacia punctulata* and *Lytechinus pictus* and 2) the cellular synthesis of RNA by eggs and embryos of *Strongylocentrotus purpuratus*.

The stimulation of polypeptide synthesis by polyribonucleotides (polyU and polyUG) was used to evaluate the rate-limiting factors involved in the increases in protein synthesis observed after fertilization and in subsequent development. In the early stages of development, the increase in the rate of protein synthesis far exceeded the increase in rate of polypeptide synthesis elicited by synthetic messenger polyribonucleotides. Either these two classes of synthesis represent differentially activated classes of ribosomes present in the egg or the more striking increase in endogenous protein synthesis is attributable to messenger RNA made available after fertilization and through early development.

Distinctly different sedimentation patterns were displayed by the rapidly synthesized RNA of three phases of development of *S. purpuratus:* The unfertilized-egg and just-fertilized-egg patterns have characteristically two

peaks (approximately 17 and 22S); a peak at approximately 10S dominates in the pregastrula embryo; and the postgastrula pattern is polydisperse. The last is coincident with a markedly enhanced synthesis of ribosomal RNA, as detected by incorporation in chase experiments.

From two lines of evidence, it is proposed that in an early phase of development, after fertilization, the reservoir of ribosomes originating in the unfertilized egg largely serves the needs of the embryo for protein synthesis. Rather than the construction of new ribosomes during this period, the production of labile or mRNA predominates, presumably to program the functional ribosomes already present. A similar emphasis on transfer RNA production occurs over this interval. In a later embryonic phase, the synthesis of ribosomal RNA increases relative to the other RNA components.

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EVIDENCE FOR MESSENGER ACTIVITY OF ALIEN RIBONUCLEIC ACID IN CHICK CELLS[1, 2]

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Our experiments explore the possibility that animal cells can ingest macromolecules—ribonucleic acid (RNA) in particular—and that these molecules can function in the host cell cytoplasm for a few hours at least. One can offer to chick embryo fibroblasts synthetic polynucleotides or RNA obtained from the rabbit, the mouse, or a bacterium, and under certain conditions demonstrate that proteins not normally made by the chick cells have been produced. Indeed the new proteins can be shown to be closely related antigenically to those characteristic of the cell from which the RNA was obtained.

We propose that what we are dealing with is messenger RNA (mRNA) (*1*, *2*), which can be likened to a tape bearing a message transcribed from the genes of the cell. This message, if it arrives intact, can function in another quite different cell lacking genes for that particular structural protein or enzyme. We think we have succeeded in this way in inducing chick cells to make bacterial or mouse proteins in response respectively to bacterial and mouse RNA.

METHODS AND MATERIALS

The preparation of primary cultures of chick embryo cells, distribution of the cells as monolayers, and the general methods employed in estimating macromolecule synthesis have been described in a previous publication (*3*). Included in the same publication are procedures for the use of radioactive amino acids, inorganic phosphate, nucleosides, and the preparation of P^{32}-labeled RNA from *Escherichia coli*.

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[5] Training Fellow of U.S. Public Health Service.

RESULTS

Chick Embryo Fibroblast in Primary Culture

Primary cultures of cells released from 10-day chick embryos by trypsinization consist primarily of 80 to 90 percent fibroblasts. In a medium of Eagle's amino acids, lactalbumin hydrolysate, and horse serum (3%), the cells adhered to glass, assumed the appearance characteristic of fibroblasts, and synthesized RNA and protein (text-fig. 1). There was no multiplication of the cells, nor evidence of deoxyribonucleic acid (DNA) synthesis, either as net synthesis or incorporation of radioactive thymidine.

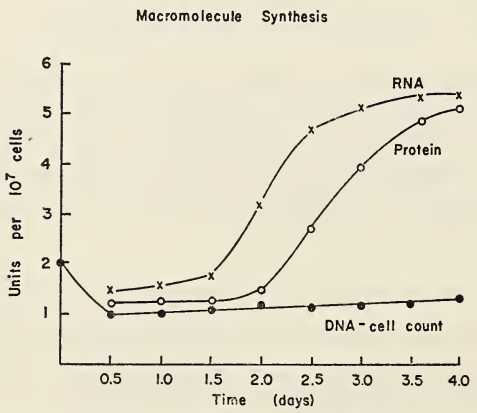
Following trypsinization the cells recovered slowly their ability to synthesize protein. The length of the restoration period and the rate of synthesis established after recovery could be controlled by adjustment of the level of serum. The higher the serum concentration the shorter was the recovery period and the higher the rate of protein synthesis after the lag (text-fig. 2).

Protein and RNA Synthesis in Serum Deprival

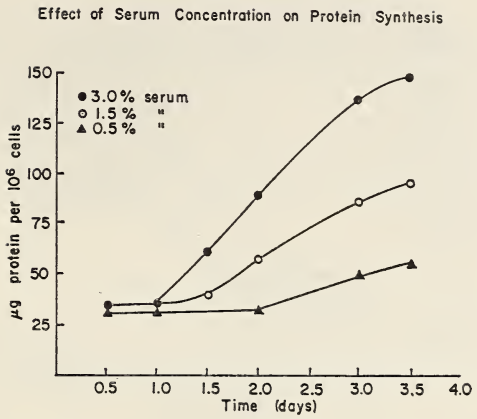
In the absence of serum no net synthesis of protein or RNA was detectable for several days (text-fig. 3). During this period, limited turnover of protein and active turnover of RNA did occur. The readdition of serum resulted in the almost immediate acceleration of protein synthesis, although the maximal rate of synthesis was not achieved for some 12 hours. Results of experiments in process indicate that cells deprived of serum are depleted both of mRNA and of ribosomes.

Stimulatory Effect of Exogenous RNA on Protein Synthesis

The period required for recovery of protein synthesis following trypsinization or serum deprival could be reduced by the addition of phenol-



TEXT-FIGURE 1.—Macromolecule synthesis. Monolayers in 3 percent horse serum. Cells from 2 bottles harvested every 12 hours. No change of medium.

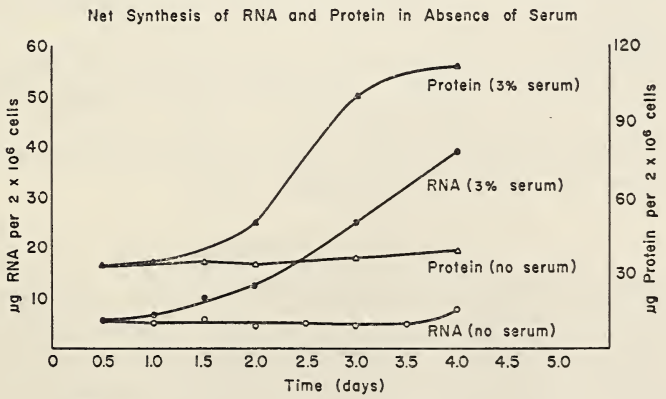


TEXT-FIGURE 2.—Effect of serum concentration on lag and rate of protein synthesis. Horse serum at concentrations indicated from 0 time. No change of medium.

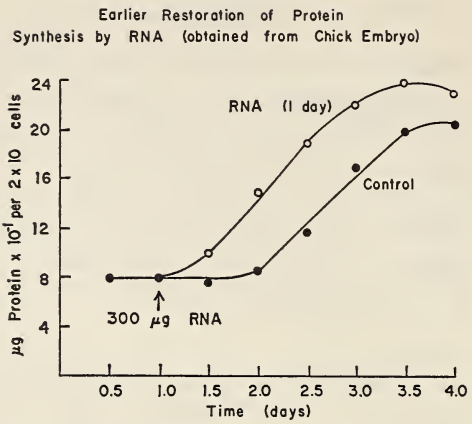
extracted RNA from several sources (text-fig. 4). We have prepared RNA from the chick embryo, mouse liver, a mouse myeloma tumor, and several strains of *E. coli*. Active preparations from any of the sources mentioned had a similar stimulatory effect that was not altered by deoxyribonuclease or trypsin treatment, but was abolished by pretreatment of the RNA extract with ribonuclease. Combinations of nucleosides in lieu of RNA failed to produce stimulation of protein synthesis.

Preparation of RNA

The influence of modifications of the phenol extraction procedure (*4*) on the yield of RNA with stimulatory activity is discussed in detail in an earlier publication (*3*). Efforts to inhibit endogenous ribonuclease activity with magnesium and sodium deodecyl sulfate (SDS) and to carry out the extractions in the cold, avoiding the use of alcohol, appeared



TEXT-FIGURE 3.—Net synthesis of protein and RNA in absence of serum. Horse serum at concentrations indicated from 0 time. No change of medium.



TEXT-FIGURE 4.—Earlier restoration of protein synthesis by RNA; 1.0 mg RNA from 10-day chick embryo added at day 1. RNA remained in contact with cells throughout experiment; 1.5 percent horse serum.

to increase the probability of obtaining "active" RNA. Even so only one of every two or three preparations proved active. Fortunately, those possessing activity were stable for several weeks if kept frozen at −20° C. All the RNA preparations obtained were contaminated to varying degrees with DNA, protein, and polysaccharide. Expressed as percentage of RNA: The protein varied from 1 to 10 percent; DNA from 0.1 to 10 percent, before deoxyribonuclease treatment; the polysaccharide from 3 to 15 percent (glucose equivalents).

Distribution of Active RNA in Cell of Origin

Phenol extraction of both the soluble and particulate fractions of bacterial lysates centrifuged at 100,000 × *g* for 90 minutes possessed activity. The soluble fraction was several times more active per mg RNA, virtually all of which activity was sedimented by further centrifugation at 100,000 × *g* for 12 hours.

Both nuclear and cytoplasmic fractions separated by centrifugation from disrupted mouse myeloma cells yielded active RNA. The cytoplasmic content was several times that of nuclei. That the so-called nuclear fraction contained virtually all the deoxyribonucleic acid suggests that the separation was in fact reasonably reliable.

Evidence for RNA Uptake

Cells exposed to P^{32}-labeled RNA could be shown to acquire cold acid-insoluble counts gradually. The definition and kinetics of uptake have been presented at length in a previous publication (*5*). Ribonuclease activity of the serum and cell surface probably constitutes a major obstacle to the penetration of intact RNA molecules. Efforts to protect RNA from degradation led to the finding that protamines and histones

(*6*) from a variety of sources enhanced its uptake. Other polybasic compounds, such as streptomycin, spermine, and spermidine, while able to retard destruction of RNA did not apparently stimulate its adsorption to or penetration of cells.

Of the information resulting from these experiments the evidence for competition for uptake between the several species of RNA and between DNA and RNA is of particular interest. This work has been presented elsewhere (*5*).

Role of Stimulatory RNA

The stimulatory function of the added RNA may represent a general effect on cell competence or a specific messenger function. The latter role would result in the synthesis of proteins identifiable as abnormal for the host cell. The protein of choice would be an enzyme not synthesized by chick cells but produced by the bacterium from which the RNA was obtained. With RNA prepared from several strains of *E. coli*, efforts were made to demonstrate directed synthesis of β-galactosidase, ornithine transcarbamylase, and the bacterial alkaline phosphatase. These attempts failed for several reasons and were complicated by the occurrence of a β-galactosidase and an alkaline phosphatase native to the chick cells; however, in both cases the chick enzyme was relatively easily distinguishable from its bacterial counterpart.

The difficulties encountered in trying to demonstrate the synthesis of a particular enzyme merit brief consideration at least. Messenger RNA constitutes perhaps 3 percent of cellular RNA in a bacterium whose messenger pool may contain 3,000 to 10,000 specific messages. There is little likelihood of a given animal cell incorporating a sufficient number of molecules bearing a given message to make detectable enzyme, particularly when the RNA offered the cell contains largely non-messenger molecules. These we have shown compete equally for uptake.

The antigenic characteristics of bacterial protein appeared to be a property offering certain advantages over enzymatic activity. The first of these is that each RNA messenger molecule contributes to the antigenic profile of the proteins. Moreover, there is considerable evidence that mutations resulting in the loss of enzyme function may alter very little the antigenic property of the protein (*7*).

"Evidence for Synthesis of Protein Antigenically Related to Bacterial Protein"

Antiserum against *E. coli* protein made in the rabbit was subsequently absorbed with chick embryo tissue to remove cross-reactive antibodies. The complement fixation test was selected as a means of detecting bacterial antigen in chick cells. Evidence suggestive of the appearance of such an antigen in cells exposed to *E. coli* RNA was obtained (table 1). Since the RNA employed in the experiment was contaminated with antigenically reactive protein, it was essential to demonstrate that the reactive protein was synthesized subsequent to the addition of RNA.

TABLE 1.—Complement fixation with protein from chick cells versus anti-coli B antibody

Cells	Day	Experiment: 1	2	3	4
Control	0	<1*	<1	<1	<1
	1	<1	<1	<1	<1
	2	<1	<1	<1	<1
RNA-treated	0	4	<1	4	2
	1	2	2	128	32
	2	16	16	64	32
	3	—	32	—	—

*Units of complement-fixing antigen per 0.5 mg of cellular protein. Values expressed as the reciprocal of the highest dilution of the protein sample showing at least 2+ complement fixation. The system contained 8 units of anti-coli B protein antiserum (absorbed with chick cell homogenate).

The addition of a radioactive amino acid to the cell culture at the time of exposure to RNA would insure that protein of a bacterial type produced in response to RNA would be radioactive. Precipitates resulting from complexes formed between antibody to *E. coli* protein and such antigenic material from the chick cells would contain radioactivity.

Unfortunately no counts were precipitated when proteins (to which unlabeled *E. coli* carrier protein was added) from cells exposed to *E. coli* RNA were reacted with antiserum to *E. coli* protein. The further addition to the reaction mixture, however, of goat antiserum to rabbit γ-globulin (in proportions selected for precipitin equivalence) resulted in significant radioactivity in the precipitates (table 2); 5 to 10 percent of the newly synthesized protein possessed the antigenic character of *E. coli* protein. The substitution of RNA from the mouse or chick embryo for *E. coli* RNA did not result in the formation of radioactive material precipitable with antiserum to *E. coli* protein. Nor did normal rabbit serum or

TABLE 2.—Bacterial protein synthesis determined by specific immunologic precipitation

Experiment No.	Treatment	Time after C^{14}-leucine (hr)	Total counts in protein (counts/min/ 5×10^7 cells)	Counts precipitated
1	None	24	8, 300	250
	RNA*	24	14, 650	1, 800
	None	36	12, 500	300
	RNA	36	22, 700	1, 600
	None	48	17, 300	600
	RNA	48	28, 500	1, 500
2	None	12	800	40
	RNA*	12	1, 250	70
	None	24	6, 300	300
	RNA	24	6, 800	280
	None	36	12, 400	250
	RNA	36	11, 300	1, 500
	None	48	15, 200	400
	RNA	48	21, 400	1, 700

**E. coli* RNA from soluble fraction; 300 μg per 10 ml of culture: remained in contact with cells throughout the experiment.

serum from rabbits immunized against influenza virus extract precipitable counts from cells stimulated by *E. coli* RNA.

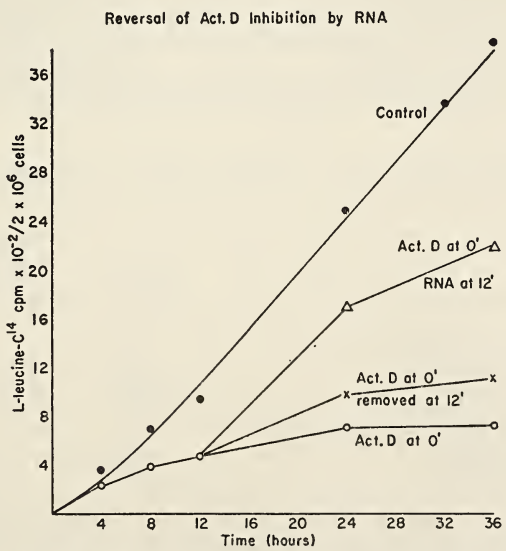
Bacterial protein synthesis was detectable only in cells exposed to *E. coli* RNA while endogenous protein synthesis was seriously curtailed, either by the low serum content of the medium or in the lag following trypsinization. No evidence of bacterial protein formation was obtained in cells actively synthesizing protein.

Actinomycin D Inhibition and Reversal by RNA

Actinomycin D (0.01 to 0.2 μg/ml) stopped RNA synthesis immediately and protein synthesis with a (mean) half-decay time of about 4 hours. The addition of *E. coli* or chick or mouse RNA as late as 18 to 20 hours after actinomycin D restored protein synthesis to at least 60 percent of its maximum rate (text-fig. 5). More thorough analysis of the kinetics of actinomycin inhibition and of reversal by RNA has been reported elsewhere (*8*).

Synthetic Polynucleotide Function in Actinomycin-Inhibited Cells

Cells whose protein synthesis had been inhibited by actinomycin D responded to the addition of polyuridylic acid by incorporation of phenylalanine but not leucine into acid-insoluble peptides (table 3). No such effect could be demonstrated in cells whose endogenous protein synthesis was sharply curtailed by the low serum content of the medium. Polyadenylic acid promoted increased incorporation of L-leucine, L-phenyl-



TEXT-FIGURE 5.—Reversal of actinomycin D inhibition by RNA. Cells in 3 percent serum 48 hours. Actinomycin D (0.04 μg/ml) added at time 0.

TABLE 3.—Polyuridylic acid stimulation of phenylalanine incorporation

	Counts in chick cells	
	L-leucine-C^{14}*	Phenylalanine-C^{14}*
Control	210	330
+ Poly U (300 μg)	230	480
+ Poly U (2 mg)	190	1, 850
+ Poly U (2 mg + 2 mg)†	260	2, 700
+ Poly A (300 μg)	320	440
+ Poly A (2 mg)	640	1, 260

*Counts per minute in 5 percent trichloroacetic acid-insoluble fraction per 10^7 cells. Cells were exposed to 0.05 μg per ml of actinomycin D for 16 hours. Either leucine-C^{14} or phenylalanine-C^{14}, both at 10^5 counts per minute per bottle (10 ml) was then added with poly U or poly A. The incubation was continued for 20 hours at 37° C. The control cells received the labeled amino acid only after 16 hours in actinomycin D.

†Two additions of poly U, the second 8 hours after the first. Poly U and poly A are expressed as the quantity added per bottle (10 ml medium).

alanine, glycine, and L-valine to about the same degree in actinomycin-treated cells. Other amino acids were not tested.

Synthesis of Bacterial and Mouse Protein by Actinomycin-Inhibited Cells

If RNA obtained from *E. coli* B or mouse myeloma cells was used to restore protein synthesis after actinomycin inhibition, some of the protein formed resembled bacterial or mouse protein. These antigenically identifiable proteins were formed only in cells exposed to the specific RNA in question (table 4). After 16 to 18 hours' exposure to 0.04 to 0.08 μg per ml of actinomycin D, C^{14}-leucine and RNA (50 to 100 μg/ml) were added to the cells. Usually a second addition of RNA was made 6 hours later, and the cells were harvested at 12 or 18 hours. The highest proportion of heterologous protein was produced in cells whose endogenous protein synthesis was most effectively suppressed. By direct precipitation with antibody, 10 to 12 percent of the newly synthesized protein proved to be

TABLE 4.—Synthesis of heterologous protein in response to bacterial and mouse RNA

	Total counts*	Counts precipitated	Counts precipitated (%)
Control	11, 900	70	0. 6
Actinomycin D (0.05 μg/ml)	250	5	2. 0
Actinomycin D + *E. coli* RNA			
Versus anti-coli serum	2, 300	290	12. 6
Versus anti-mouse serum	2, 300	11	0. 5
Actinomycin D + mouse myeloma RNA			
Versus anti-mouse serum	1, 600	180	11. 3
Versus anti-coli serum	1, 600	4	0. 25

*All values expressed for 10^6 cells. Cells were exposed to actinomycin D for 16 hours at 37° C at which time both leucine and the RNA preparations were added. Another 12-hour incubation was permitted before harvesting the cells. The proteins were released by sonication and reacted with the antisera indicated. A small amount of carrier, nonradioactive protein of the appropriate variety was added to the antigen-antibody reaction mixture. The counts are those contained in the thrice-washed precipitates.

either bacterial or mouse in the best experiment. Precipitation of the complex of protein and rabbit antibody (anti-*E. coli* protein or anti-mouse γ-globulin) with goat antiserum to rabbit γ-globulin yielded values of up to 25 percent on the same experimental material.

DISCUSSION

Evidence has been presented that chick embryo fibroblasts can synthesize limited amounts of bacterial or mouse proteins after exposure to bacterial or mouse RNA. Although the RNA appears to be taken up equally well under a variety of conditions, only cells in which endogenous protein synthesis has been curtailed respond by making heterologous protein. Suppression of protein synthesis can be achieved either by reducing the serum content of the medium or by the use of actinomycin D. RNA has been shown to stimulate protein synthesis in both of these conditions.

Since actinomycin D inhibits the synthesis of RNA by the DNA-dependent polymerase (*9–11*), protein synthesis grinds to a halt when mRNA is exhausted. Exogenously supplied messenger might be expected to find its way to the ribosomes and to reinitiate protein synthesis. The experimental findings are consistent with such a proposal in that exogenous RNA reinitiates protein synthesis in cells inhibited by actinomycin D. Moreover, the relative activity of different RNA preparations in reversing actinomycin inhibition correlates well with their ability to stimulate *in vitro* amino acid incorporation into acid-insoluble peptides (*12*). Synthesis of proteins reactive for antibody to known proteins does not constitute proof that the molecule synthesized is identical to that used as antigen in the production of antibody. Far more convincing would be the isolation and physical characterization of the protein synthesized in the chick cell. If the protein in question were also an enzyme, the integrity of its catalytic function would be an additional and sensitive measure of its similarity to the natural protein. Efforts to demonstrate enzyme synthesis following the use of *E. coli* RNA are in process.

The implications of the functioning of exogenous message in the cell cytoplasm may be far-reaching. The effect lasts but a short while and could be looked upon as a transient phenotypic (functional, nongenetic) transformation. Such transformations may be relatively common among cells in an animal and may play a significant role in, for example, the mutual compatibility of different cell types that have common frontiers in an organ or tissue. Different types in direct contact may very well be phenotypic transformants by virtue of a continuous exchange of functional RNA. This would mean that each is so modified as to be much more similar to the neighboring type than other cells more distant from the frontier. There need be nothing mysterious about the contribution of such a mechanism to surface compatibility, since the protein portion of the cell membrane is probably synthesized under the direction

of mRNA. Furthermore it is well established that viral mRNA can modify early in the infectious cycle the antigens of the cell membrane (*13*).

SUMMARY

Evidence has been presented that synthetic polynucleotides (polyuridylic and polyadenylic acids) and RNA obtained from mouse and bacterial cells can penetrate chick embryo fibroblasts.

Under certain conditions the cells respond to the exogenous polynucleotides or RNA by synthesizing polypeptides or proteins that are related antigenically to those of the cells from which the RNA was obtained. Both bacterial and mouse proteins appear to have been synthesized in response to the presence of bacterial (*E. coli*) and mouse RNA, respectively.

If exogenous RNA is to function, the endogenous protein synthesis must be seriously curtailed. This can be accomplished with a low serum concentration in the medium or by pretreatment of the cells with actinomycin D.

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MODE OF ACTION OF THE EXOGENOUS RIBONUCLEIC ACID IN CELL FUNCTION [1,2]

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Nuclear transplantation experiments have shown that during embryogenesis nuclei undergo differentiation. At the cellular level, nuclear differentiation can best be seen in the variety of blood cells originating from the common cell type, hemocytoblasts. Biochemically speaking, special enzymes are found in specific types of nuclei (*1*). However, a direct correlation between fine structure and cellular function is shown by the observation that the "puffing" pattern of the insect polytene chromosome varies according to the types of the 4 somatic tissues studied (*2*, *3*). The occurrence of "puffing" has been generally considered as differential "activation" of the gene in various types of cells during and subsequent to the act of determination.

Cell determination is the consequence of a process called embryonic induction. Thus the causal factor of embryonic differentiation is also responsible for the activation of the genes. Biochemical study of induction has demonstrated that ribonucleoprotein plays a key role in differentiation. Further analyses have shown that ribonucleic acid (RNA) can initiate urodele gastrula ectoderm to develop into highly specific structures. The type and pattern of the structure resemble embryologically the RNA tissue source (*4*). In the chick embryo, RNA isolated from 11- to 13-day chick brains induces the epigenesis of a double brain and/or excessive growth of the brain in *in vitro* blastoderm. Notochord RNA causes the enlargement of the host notochord and/or its duplication. Liver RNA has no effect (*5*, *6*). Furthermore, when the RNA-treated mouse ascites cells are tested for the biosynthesis of specific proteins, it has been shown that RNA isolated from liver dictates the type of protein to be synthesized intracellularly and thus carries the genetic code of the liver cells. Serum albumin is one of the liver-specific proteins which cannot be produced under our experimental conditions by *in vitro* cultured mammalian cells. After treatment with liver RNA, however, they acquire the specificity to manufacture it (*7*). In addition, these liver

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[3] I wish to thank Carl Cordova and Phyllis Budin for their expert assistance.

RNA-treated cells are capable of synthesizing such liver-specific enzymes as trypotophan pyrrolase and glucose-6-phosphatase (*8*), and arginase, ornithine transcarbamylase, and fructose-1,6-diphosphatase (*9*). Similarly pancreas RNA has recently been shown to increase the production of amylase by 250 to 300 percent (*10*).

DISTRIBUTION OF C^{14}-RADIOACTIVITY IN THE C^{14}-RNA-TREATED ASCITES CELLS

The success in adopting mouse ascites cells (both Lettre Ehrlich and Nelson strains) for the study of RNA function has finally overcome my long-standing difficulty in obtaining sufficient quantity of experimental material for biochemical analysis. As a logical subsequent step to the observations mentioned, experiments were designed to explore the *mode* or *mechanism* under which foreign RNA acts upon the cells. The data used in this discussion are drawn from both published and unpublished materials.

A prerequisite for the analysis of the RNA action is the entrance of C^{14}-RNA macromolecules into the cells during the treatment process. The entrance was demonstrated by both autoradiographic method (*11*) and biochemical analysis (*7*, *12*). Autoradiography revealed data of qualitative nature, but unfortunately was not sensitive enough for any definitive quantitative information. Consequently, the C^{14}-RNA-treated cells were separated into nuclear and cytoplasmic fractions according to the procedure of Hudack and Baker (*13*). The cytoplasmic fraction was further fractionated by differential centrifugation into mitochondrial, microsomal, the *p*H 5 enzyme, and the supernatant.

The nuclear fraction was divided according to Sibatani *et al.* (*14*) into chromosomal, nucleolar, and extractable components and also, according to Rees *et al.* (*15*), into nucleolar, chromosomal, heterochromatin, and supernatant components. Carbohydrates and lipoids were removed from each component, and radioactivity was counted (cpm/mg protein-cpm/mg of nucleic acid-protein). The counts of radioactivity were used as a measure of the amount of exogenous C^{14}-RNA attached to that particular fraction. Table 1 [average of 2 determinations (*16*)] shows that the specific activity of C^{14} is high, as expected, in the *p*H 5 enzyme fraction. The remarkable thing is that within the nucleus a higher count was found frequently in the chromosomal rather than in the nuclear extractable fraction (a combination of nuclear-microsome and nuclear sap). Similar results were obtained with the 4 nuclear fractions. Therefore, the exogenous RNA seems to have some sort of affinity for chromosomal material. In this sense, I have described RNA as a genotropic substance.

The nature of the association between C^{14} activity and chromosomal material was analyzed with the aid of ribonuclease, deoxyribonuclease, and a combination of both enzymes (*16–18*). An average of 90 to 97 percent of the radioactivity was eliminated by digestion with ribonuclease,

TABLE 1.—Radioactivity distribution in different cellular components (cpm/mg protein) of the C^{14}-liver-RNA-treated Ehrlich ascites cells*

Time of treatment (hrs)	Nuclear fractions			Cytoplasmic fractions			
	Chromosomal	Nucleolar	Extractable	Mitochondrial	Microsomal	pH 5 Enzyme	Supernatant
2	148	64	134	102	117	375	32
24	430	150	161	174	223	856	111
Control†	105	39	151	87	92	1,155	164

*Average of 2 determinations.
†C^{14}-liver-RNA added to the medium used in fractionation (water-alcohol mixture).

10 to 18 percent by deoxyribonuclease, and 99 to 100 percent by both enzymes. After treatment with deoxyribonuclease, the loss of C^{14} activity might be due either to the impurity of deoxyribonuclease or to the presence of C^{14}-containing molecules in the deoxyribonucleic acid (DNA) chemical configuration. The latter could be the result of either the utilization of degraded C^{14}-RNA molecules as precursor for DNA synthesis during DNA biosynthesis (*11*) or the conversion of some C^{14}-RNA molecules into C^{14}-DNA. Judged from the constancy of ultraviolet absorption at 260 mμ in RNA solution containing deoxyribonuclease, the impurity, if present, seemed to have no hydrolytic property on the RNA molecule. Should the reported similarity of C^{14} ratios of bases between C^{14}-RNA isolated from the C^{14}-liver-RNA-treated cells and C^{14}-liver-RNA represent configurational identity between these two C^{14}-RNAs, one could use this similarity as evidence against RNA degradation and subsequent utilization of its hydrolytic products as precursors for new DNA biosynthesis. This favors the conversion hypothesis as a likely candidate. However, additional data are needed before final conclusion can be drawn.

RNA AND PROTEIN BIOSYNTHESIS OF TREATED CELLS

The attachment of the exogenous RNA to chromosomal material described suggests that RNA may actually regulate the DNA function in a fashion similar to that of "puffing" in polytene chromosomes (*2*, *17*), or of arginine-rich histone (*19*, *20*). The RNA effect on RNA and protein biosynthesis was first shown by the following analyses:

RNA Biosynthesis

Both the RNA-treated and untreated ascites cells were used for the study of RNA synthesis with H^3-uridine as precursor. The newly synthesized RNA is H^3-labeled (H^3-RNA) to distinguish it from the liver-C^{14}-RNA. Normally, however, the RNA used in the treatment of the cells was cold. The treated cells were suspended in 10 volumes of

TABLE 2.—Amount of H^3-RNA synthesized in different cell components of the C^{14}-liver-RNA-treated and untreated ascites cells*

Series of experiments and treatment of cells	Nuclear fractions			Cytoplasmic fractions			
	Chromosomal I	Nucleolar II	Extractable III	Mitochondrial IV	Microsomal V	pH 5 Enzyme VI	Supernatant VII
1. a. Ascites cells in saline overnight—control.	529	850	5	155	32	37	9
b. Ascites cells in liver-RNA solution for 2 hours.	203	491	97	247	48	39	9
c. Ascites cells in liver-RNA overnight.	370	562	114	324	52	59	26
2. a. Control as 1a	421	309	221	12	118	2	25
b. Experimental as 1c	82	152	219	132	141	5	14
3. a. Control as 1a	1,083	1,787	183	479	20	90	11
b. Experimental as 1c	935	1,023	371	387	445	261	17

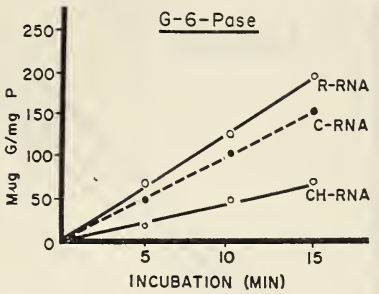
* Expressed as counts of radioactivity (cpm/mg protein). Incubation time was 25 minutes in all, except for 60 minutes in series 3.

cold medium 199 with 1 μc per ml H^3-uridine added and then incubated at 37° C for 25 minutes. The amount of H^3-RNA associated with various cell components had already been analyzed (*16*). The results (table 2) showed clearly that RNA treatment preferentially reduced the H^3-RNA in the chromosomal and nucleolar fractions and conversely increased the amount linked with *all* other components (nuclear sap and all cytoplasmic particulates). Therefore, the RNA-induced augmentation of cytoplasmic RNA is due to an elevated activity of the DNA-dependent RNA biosynthesis. In other words, the exogenous RNA has somehow activated the DNA function.

Protein Biosynthesis

The correlation between the activated DNA function and the increase of cytoplasmic RNA suggests that the latter might be contributed by the so-called messenger RNA (mRNA). The functioning of this mRNA should lead to the production of specific enzyme or protein. Among the specific enzymes studied, glucose-6-phosphatase (G-6-Pase) is now used as an example of analysis. Text-figures 1 and 2 show, respectively, the kinetics and specific activity of the enzyme, G-6-Pase. The liver RNA-induced formation of G-6-Pase is sensitive to ribonuclease, actinomycin D, 5-azauridine (*16*), and chloramphenicol (text-fig. 3), but indifferent to penicillin-streptomycin or kanamycin [500 μg/ml, *see* (*21*)]. The release of inorganic phosphorus (Pi) by G-6-Pase was also determined (*8*). With glucose-6-phosphate as substrate, the enzymatic liberation of Pi and glucose (G) has been taken as evidence for the activity of G-6-Pase (text-fig. 4). Furthermore, the G-6-Pase activity in our system can be inhibited by the addition of glucose (150–300 μg/ml).

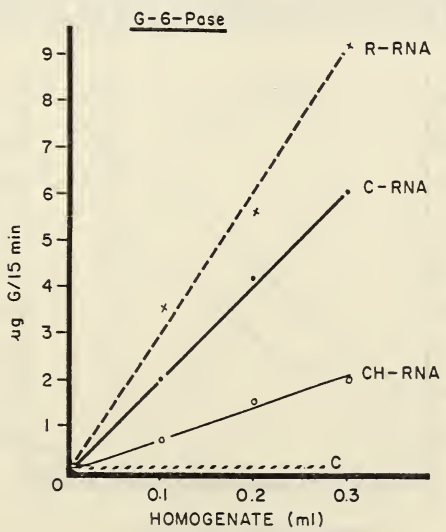
RNA used in this work was prepared from liver. Separation into microsomal and soluble fractions was accomplished by chemical means



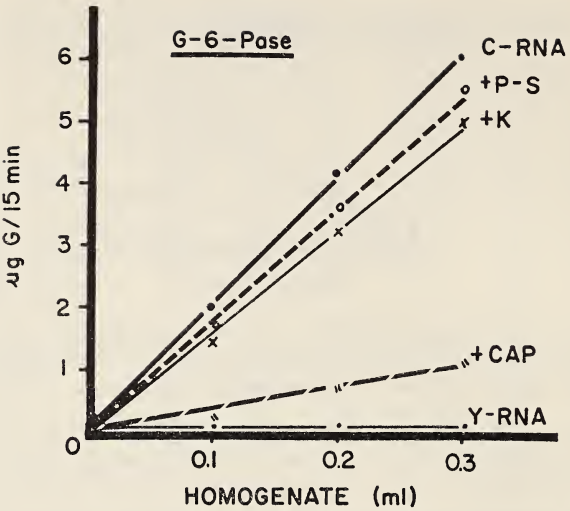
TEXT-FIGURE 1.—Time course of G-6-Pase activity in the Ehrlich ascites cells treated with rat (R), calf (C), and chicken (CH) liver RNAs. The activity is expressed by the amount of glucose released (mμg glucose/mg protein).

(*8*), sucrose gradient (*22*), column chromatography with methylated serum albumin (*23*), and ecteola cellulose column chromatography (*24*). The ratio of specific activity of G-6-Pase initiated by the soluble and microsomal RNA was about 3 (text-fig. 5). Further fractionation of the soluble RNA resulted in 4 subfractions. Their functional capacity for inducing the biosynthesis of G-6-Pase (text-fig. 6) increased arithmetically according to the sediment coefficient, S_{20} [I, 3.7; II, 4; III, 4.3; and IV, 4.6 (*24*)]. Study of the nuclear fractions is the thesis subject of Mr. Philip Nash, a graduate student of this laboratory.

The concept, that the type of proteins synthesized by the RNA-treated cells is specific to the tissue source of donor RNA, has recently gained support from studies on the induced formation of abnormal hemoglobin

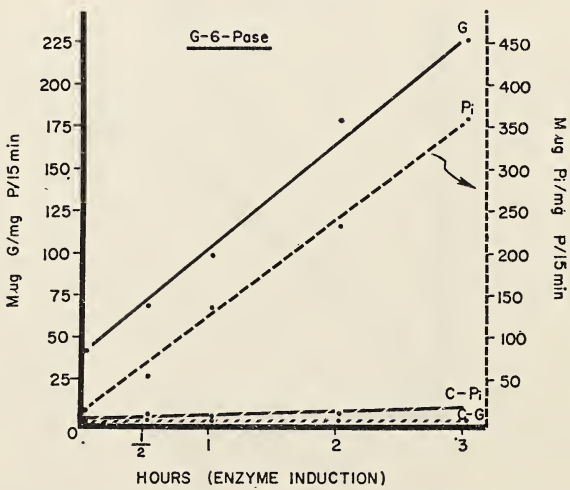


TEXT-FIGURE 2.—Concentration effect of the enzyme in ascites cell homogenate on G-6-Pase activity. The cells used and abbreviations are the same as text-figure 1.

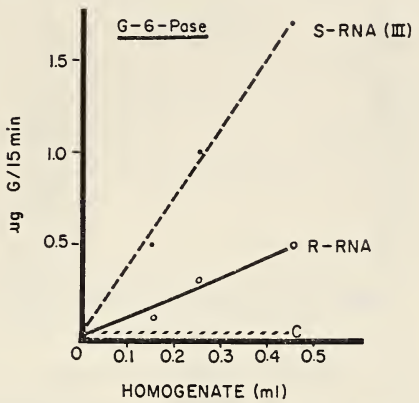


TEXT-FIGURE 3.—Effect of antibiotics on G-6-Pase biosynthesis of the calf liver RNA (cRNA) treated Ehrlich ascites cells. P-S, penicillin-streptomycin (Microbiological Associates, Bethesda, Md.); K, kanamycin (Parke and Davis, Detroit, Mich.), 500 μg per ml; CAP, chloramphenicol (Parke and Davis); and yeast RNA, control. The inhibitory effect of CAP is striking.

in bone marrow cells (*25*), of tobacco mosaic virus-protein in cell-free systems of *Escherichia coli* (*26*), *E. coli* protein in chick fibroblasts (*27*) (and the paper discussed during this Symposium), antigenic protein of leukemic cells in human amnion cells (*28*), the RNA transfer of transplantation immunity (*29*), the pancreas RNA-increased biosynthesis of amylase (*10*), the kidney RNA-increased production of amino acid oxidase (AAO)



TEXT-FIGURE 4.—Time course of G-6-Pase activity as measured by the liberation of glucose (G) and inorganic phosphorus (Pi).

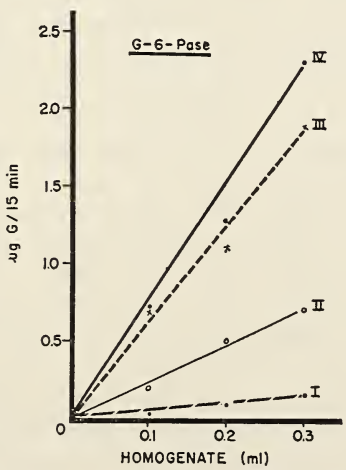


TEXT-FIGURE 5.—Differential potentiality of soluble (s) and microsomal (r) RNA in the induced biosynthesis of G-6-Pase.

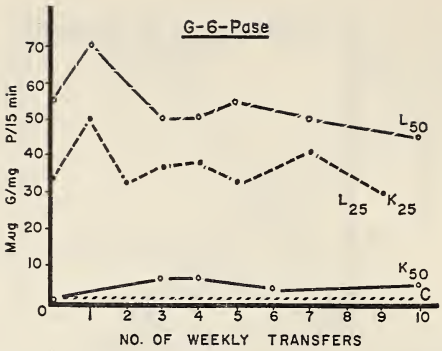
and leucine amino peptidase (*30*), RNA-induced synthesis of tryptophan synthetase in *Neurospora crassa* (*31*), and the f2/RNA-induced synthesis of f2 coat protein (*32*).

STABILITY OF ACQUIRED BIOSYNTHETIC CAPACITY

Early discussion of RNA biosynthesis has revealed that newly synthesized RNA is decreased in chromosome and nucleolar components in the RNA-treated cells and this reduction is accompanied by an increase of RNA in every cytoplasmic component. In view of the nuclear origin of cytoplasmic RNA, the increase indicates that RNA treatment has

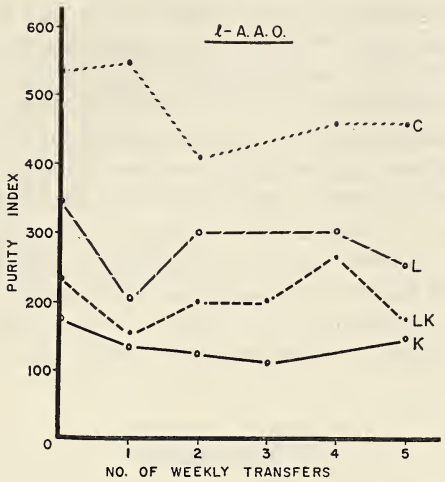


TEXT-FIGURE 6.—Differential capacity of the 4 sRNA subfractions in the induced biosynthesis of G-6-Pase.



TEXT-FIGURE 7.—Acquired activity of G-6-Pase in Ehrlich ascites cells treated with liver RNA. These cells were then subjected to weekly serial transfer. L, liver, and K, kidney; the number indicates the optical density equivalent of RNA used in the initial treatment of the cells.

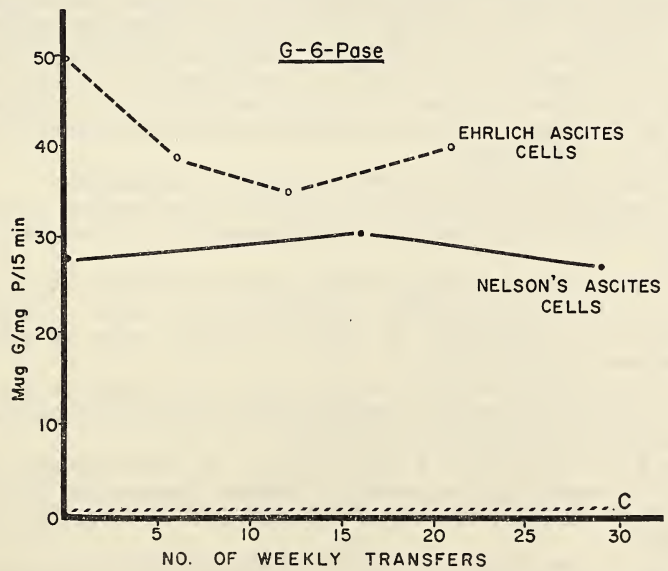
brought changes of nuclear activity prior to the acquisition of new machinery for specific enzyme formation. An immediate question is the stability of the altered nuclear function. The kinetic study of specific enzyme induction has shown that the rate of increase is linear (text-fig. 1) or sigmoid, depending on the kind of cells used. That the increase of G-6-Pase does not need RNA during the period of enzyme induction shows some stability of the induced changes. To analyze this question further, two lines of approach were adopted. Both were designed on the basis that if the RNA-induced enzyme formation was stabilized, the acquired capacity should be retained through serial transfers of the RNA-treated cells. First, RNA from calf liver and calf kidney was used separately to treat Ehrlich ascites cells. Subsequently they were injected intraperitoneally into mice (15–20 g). The tumor produced was maintained through successive weekly transfers in the abdomen of Swiss-Webster mice. Cells used for each transfer were also tested for specific enzyme activity. Text-figures 7 and 8 illustrate, respectively, the enzyme activity produced by liver and kidney RNA. Second, the treated Ehrlich and Nelson ascites cells were transferred in the same fashion as the untreated stock. Beginning on the 3d day after injection, each mouse received 3 to 5 daily intraperitoneal injections of 0.5 ml saline (0.9% NaCl) containing calf liver RNA (optical density 100/ml). Ehrlich cells thus treated were collected and used for subsequent weekly transfers. The Nelson cells were again treated with RNA in the cold, overnight, and then used for transfers. Two more intraperitoneal injections of RNA were given. The maintenance of G-6-Pase activity in these transferred cells is further shown in text-figure 9. The acquired biosynthetic activity has been maintained, up to the present writing, through 21 and 29 weekly transfers. If 20 hours were the average generation time of these ascites cells, 29 weeks would amount to approximately 244 generations. During this period these cells have not lost *in vivo* their learned properties for biosynthesis.



TEXT-FIGURE 8.—Acquired activity of L-amino acid oxidase (L-AAO) in Ehrlich ascites cells treated with liver, liver and kidney, and kidney RNA.

SUMMARY

Freshly prepared C^{14}-RNA was used to treat mouse ascites cells. The location of the C^{14}-RNA in the treated cells was analyzed by cellular fractionation. Data obtained from two procedures of isolation showed that within the nucleus the highest activity was in the chromosomal component. New RNA biosynthesis, as indicated by the incorporation



TEXT-FIGURE 9.—Sustained activity of G-6-Pase in 2 strains of mouse ascites cells that have undergone weekly serial transfers.

of H^3-uridine, was decreased in the chromosomal and nucleolar fractions of those cells treated with C^{14}-RNA. Accompanying this decrease was an increase in H^3-RNA in all remaining cell components. Since RNA biosynthesis is DNA-dependent and the newly synthesized RNA of the cytoplasm is responsible for the enzyme formation, it appears that the exogenous RNA affects primarily the nuclear (DNA) function. Direct support to this concept comes from studies on the stability of the RNA-induced acquisition of biosynthetic capacity. Up to this writing, the acquired capacity has been maintained through 21 serial weekly transfers in the RNA-treated Ehrlich ascites cells and 29 in the Nelson cells. Assuming that the generation time of these ascites cells is 20 hours, 29 transfers would amount to approximately 244 generations.

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REGULATION OF ENZYME SYNTHESIS AND ENZYME ACTION

Chairman: Boris Magasanik

SOME STUDIES OF ENZYMES IN CULTIVATED HUMAN CELLS [1, 2]

ROBERT DEMARS,[3] Department of Medical Genetics, University of Wisconsin, Madison, Wisconsin

THE considerable degree of environmental control gained by cultivation of human cells *in vitro* has made them prime subjects for the quantitative study of factors that influence enzyme levels. An important part of the work that has been done concerns the relation between the amount of an enzyme found in the cells and a genetic variable, such as a variation in chromosome number or the presence of a mutant gene. In a general sense, these are studies of gene dosage effects and I will discuss two such studies that are in progress in my laboratory.

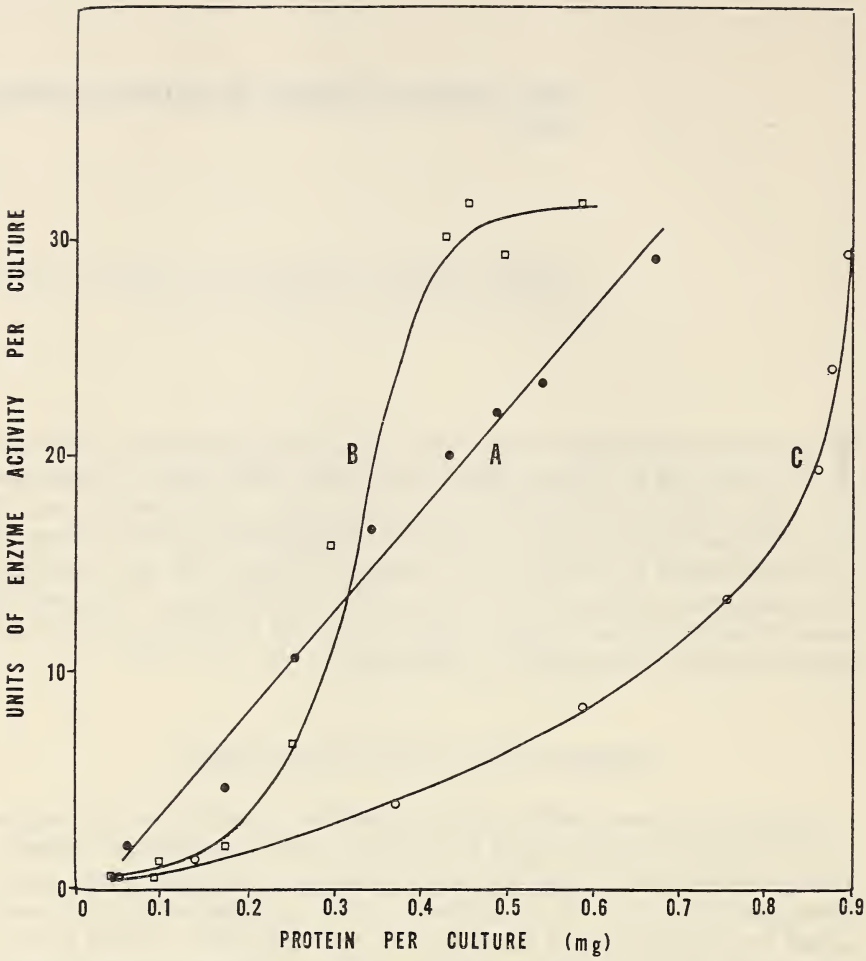
STUDIES WITH ANEUPLOID CELLS

The first of these involves the comparative quantitation of enzyme levels in diploid cells cultivated from humans with normal sets of chromosomes or sets deviating from the norm by having a particular chromosome present partially or wholly in triplicate. Three well-defined types of such partial or total autosomal trisomy have been described. These involve chromosome 18 (*1*), a D chromosome (*2*), and the mongolism trisomy chromosome (*3*). The simple motivation of our work has been to detect some consistent influence of third copies of genes on these chromosomes on the amounts of various enzymes present in the cultivated cells. Hopefully, such relations would contribute to a cytological foundation for genetic analysis with somatic cells. We have no way of selecting particular enzymes that are likely to reveal effects of gene dosage and, for the time being, our criteria of selection are purely practical: that the enzyme activity is amenable to simple colorimetric assay and that the assay can be executed with small quantities of cells. The latter criterion is related to the necessities of minimizing the number of individual cultures used and of com-

[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27-30, 1963.

[2] Paper #949 from the Division of Genetics, University of Wisconsin, Madison, Wisconsin; work was supported by grants GM06983 and GM08217 from the National Institute of General Medical Sciences, National Institutes of Health, Public Health Service.

[3] Studies of acid phosphatase and electrophoretic variants of glucose-6-phosphate dehydrogenase were made in collaboration with Dr. Jean M. Marsh and Dr. Walter E. Nance, respectively. The study of β-glucuronidase was performed by Dr. Jessica A. Gorman while she was a graduate student in the laboratory.



TEXT-FIGURE 1.—Increase in enzyme activities during the growth of cells cultured from human skin. Acid phosphatase: –●–; β-glucuronidase: –○–; glucose-6-phosphate dehydrogenase (G6PD): –□–. The 3 curves were derived from separate experiments. Cells were the fusiform variety commonly obtained from skin biopsies. Replicate cultures were sampled at intervals during growth for determinations of enzyme activity and protein content. Acid phosphatase activities were determined with acetone-fixed cells on 18 mm diameter coverslips, overlayed with 1.0 ml of reaction mixture containing 3×10^{-4} M *p*-nitrophenyl phosphate in 0.4 M sodium citrate buffer at *p*H 5.0. After 60 minutes at 37° C, 4.0 ml of 0.1 N NaOH were added and the optical density was determined at 410 mμ. The optical density of 1 μmole of *p*-nitrophenol with this procedure was 3.0. β-Glucuronidase activity was determined with acetone-fixed cells grown in petri dishes 30 mm in diameter. The reaction mixtures were 0.6 ml of 0.1 M sodium acetate buffer at *p*H 5.0 containing 2×10^{-3} M phenolphthalein glucuronide. After appropriate incubation at 37° C, 0.6 ml of cold 1 M glycine buffer at *p*H 10.5 was added and the optical density at 555 mμ was determined. The optical density of one μmole of phenolphtalein with this procedure was 22.6. G6PD was determined with cells that were sonically disrupted in water containing 2×10^{-5} M TPN. The rate of change of optical density at 340 mμ and 37° C was determined with 1 cm cuvettes and a 1.0 ml mixture

paring as many different normal and abnormal strains of cells as possible in each experiment. Finally, since the scale of these comparative experiments must be large, we work with enzyme activities that are accurately determinable with minimal manipulation of the cells. A final consideration emphasizes the need for mass production methods at certain stages of the work. We expected the relation between the amount of an enzyme activity and the number of cells or the total amount of cell protein might vary during the culture cycle. Therefore, comparison of enzyme activities in different strains of cells has often involved repeated determinations during growth.

Solutions to these problems are exemplified in quantitative studies of acid phosphatase activity. In these experiments several coverslips (18 mm diameter) were immobilized in the bottoms of plastic petri dishes with tiny flaps of plastic. Replicate aliquots of the appropriate cell suspensions were introduced into the dishes. The cells attached to and proliferated on the glass. The coverslips then bore replicate samples of the cell population in each dish. Daily, during the growth of the cultures, coverslips were removed from the dishes, rinsed in 0.9 percent NaCl, and fixed in acetone at room temperature. Two coverslips from each dish were used for determinations of acid phosphatase activity and two were used in the determination of total cell protein (*4*). The enzyme assays were executed by placing the dried coverslips in flat-bottomed vials and overlaying them with reaction mixture. After suitable incubation the reactions were stopped, and the amount of enzymatically liberated product determined.

The relation between the increase in acid phosphatase activity and total cell protein is exemplified in curve A of text-figure 1. It is evidently a simple, straight line relation and this has been true, without exception, in studies of at least 20 different cell strains and many more than 20 individual experiments. The slope of such straight lines is the specific activity (units of enzyme activity per unit of total cell protein) of newly formed protein and can be used to express the acid phosphatase phenotypes of each cell strain. When environmental conditions are carefully controlled, these slopes are so reproducible that we can say the specific activities of euploid and trisomic cells of the three sorts referred to are indistinguishable from one another (*5*). Text-figure 2 is a cursory illustration of this point.

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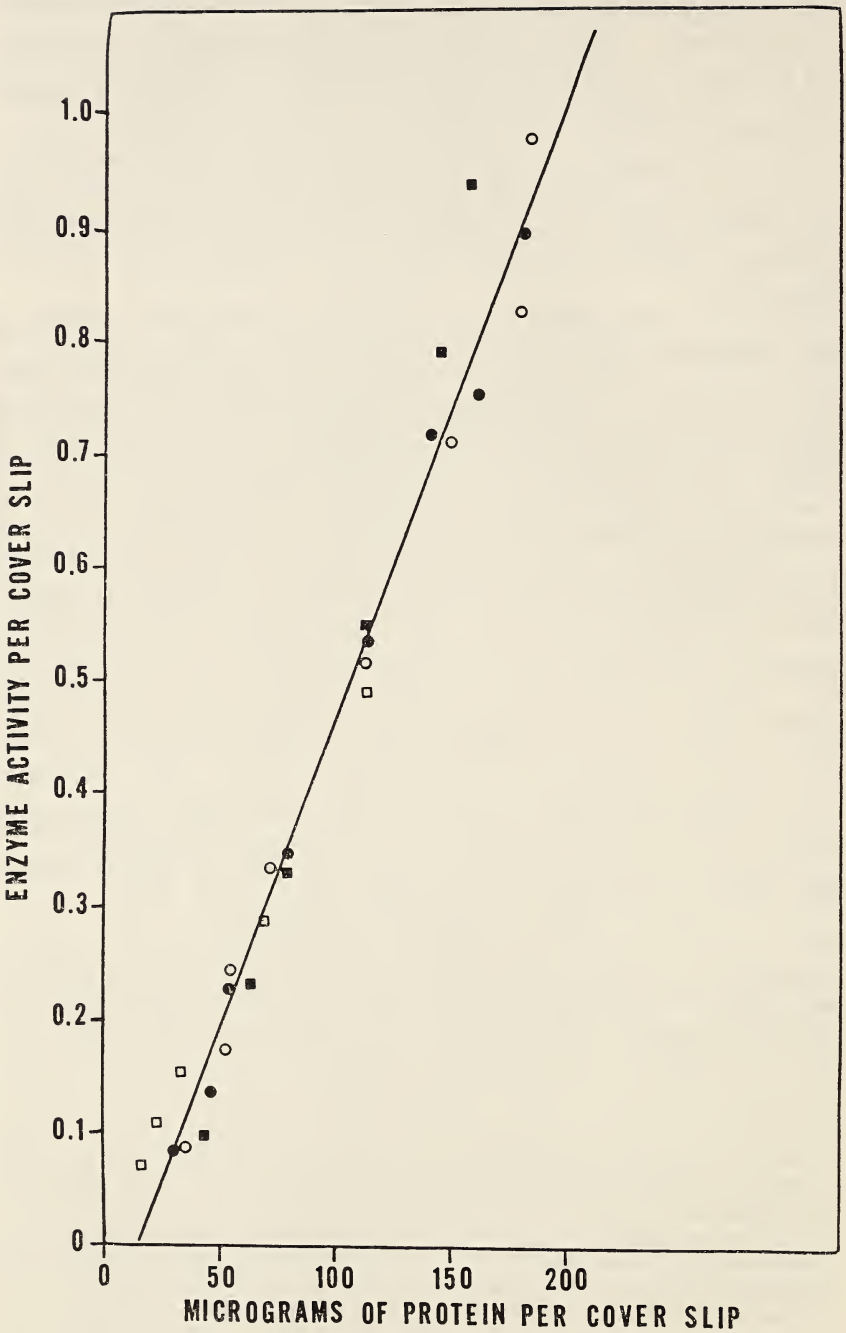
containing Tris, 0.1 M, *p*H 8.0; TPN, 2×10^{-4} M; $MgCl_2$, 5×10^{-4} M. In addition the mixtures contained either 6-phosphogluconate (6×10^{-3} M) alone or plus glucose-6-phosphate (6×10^{-3} M). A substrate-free blank containing cell extract was used. The difference between the rate with both substrates and with 6-phosphogluconate alone was taken as the rate for G6PD. In this system 1 μmole of TPNH had an optical density of 6.2. For comparison, all values shown in the figure have been normalized to correspond to the population in a 30 mm diameter petri dish. On the scale used for expressing enzyme activity with the procedures outlined above: One unit of acid phosphatase activity means the liberation of 30 mμmoles of *p*-nitrophenol per hour. One unit of β-glucuronidase means the liberation of 1 mμmole of phenolphthalein per hour. One unit of G6PD means the appearance of 3 mμmoles of TPNH per minute.


ENZYME ACTIVITY PER COVER SLIP
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
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0
0
50
100
150
200
MICROGRAMS OF PROTEIN PER COVER SLIP

The formation of acid phosphatase at constant specific activity throughout growth indicates that fairly reliable phenotypic determinations could be made with one or two samplings during the culture cycle. This is the practice in much work done with enzymes in cultured cells, and my comments about β-glucuronidase activity indicate that it is not always a reliable one—for the relation between the increase in β-glucuronidase activity and the increase in total cell protein is not a simple one. Curve C of text-figure 1 shows that the specific activity of this enzyme varies significantly during growth. Initially, the relative increase in total cell protein is greater than that in enzyme activity and the specific activity sometimes drops to one third or one fourth the initial value during the first several days. As the populations become denser and the over-all growth rate declines the relation between the two parameters is reversed. The specific activity rises and approaches its initial value. In some experiments increases of 50 to 100 percent in enzyme activity occur without a change in the net protein content of the cultures. The general shape of the curves is the same for all strains tested, whether euploid or aneuploid. This shape has precluded the use of a single numerical expression for the β-glucuronidase phenotype of a strain of cells. Single determinations, made arbitrarily during growth of the cultures, could be misleading for purposes of interstrain comparisons, except where very large differences in activity distinguished the strains. We have resorted to making the repeated determinations depicted here and comparing the strains at several, comparable population densities. When this is done no significant effect of the 3 defined autosomal trisomies on β-glucuronidase levels is detected (*6*).

Despite this negative result, the shape of the curves relating glucuronidase increase to protein increase has intrinsic interest. The disproportionately large rise of enzyme activity in dense cultures, where cell number and total protein had become almost constant, suggested that enzyme formation might be responding to either increased or decreased concentrations of components of the culture medium. It had previously been reported that medium in which cells had already grown evoked large increases in the alkaline phosphatase activity of cultured cells (*7*). This was true for β-glucuronidase, too, though the maximum increases in specific activity were but fivefold or sixfold. Repeated determinations showed that the use of "conditioned" medium or medium with 5 percent instead of 15 percent serum supplement brought net protein increase to a virtual halt, even in cell populations of submaximum density. β-glucuronidase activity continued to increase for at least 5 days, in this circum-

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TEXT-FIGURE 2.—Acid phosphatase activity of cells cultivated from human skin. Euploid: –○– (slope = 0.00535). Trisomic for chromosome 18: –●– (slope = 0.00534). Trisomic for a D chromosome: –□– (slope = 0.00425). Trisomic for the chromosome causing Down's syndrome (mongolism): –■– (slope = 0.00645). Line is the average for this group of strains and has the slope 0.00535. Experimental format is described in text and the procedure for determining enzyme activities is described in the legend of text-figure 1.

stance, while acid phosphatase activity remained constant. The increase could be completely suppressed by puromycin. Finally, all attempts to detect "masked" enzyme activity have failed (*6*). The results are consistent with the notion that the proteins of the static cell populations are actually in a dynamic state. Here, the net amount of some proteins is stabilized while, for others, such as β-glucuronidase, it may increase. The dynamic state of proteins in HeLa cells has been demonstrated by Eagle and Piez (*8*) using radioactive amino acids and has occurred to a similar extent in the diploid cells used in our work (*6*). There is evidence the turnover process detected by the study of total cell protein is reflected in the behavior of specific enzymes. We have no idea what factors regulate the behavior of β-glucuronidase, but Cox has shown (*9*) that cysteine is a repressor of alkaline phosphatase formation in cultures of diploid cells similar to ours. The repressive effects of cysteine were not demonstrated by studying enzyme activities during increase of the cells in media containing or lacking cysteine, for the cells fail to increase when that amino acid is absent. Instead, the cells were placed in a simplified maintenance medium in which the cell populations remained virtually constant. Enzyme activities after sojourn in the presence or absence of cysteine were then related to the initial levels, *i.e.*, the repressive effects of cysteine were detected as a decrease in phosphatase activity already existing rather than as prevention of an increase in activity. The results could be interpreted in terms of a breakdown of alkaline phosphatase molecules. Resynthesis approximately equaled breakdown when cysteine was absent, but was suppressed in its presence causing a net loss of enzyme activity. Similar results were obtained with the γ-glutamyl transferase of HeLa cells several years ago (*10*). Glutamine is a specific repressor of transferase formation during growth, when present at concentrations as low as 2×10^{-5} M. Cells grown in medium in which glutamine is replaced by glutamic acid form the enzyme at a rate approximately 15-fold greater than when glutamine is present. Populations of cells grown in the absence of glutamine and having maximal levels of transferase were placed in a medium devoid of 3 essential amino acids. The net protein content of the cultures remains almost constant for several days in such medium, but amino acid residues in preformed protein exhibit an active turnover involving about 0.5 to 1.0 percent of the residues per hour (*8*). In medium containing glutamine (2×10^{-3} M) the activity after 48 hours was only 15 percent of the original, while 75 percent of the activity remained in medium in which glutamic acid replaced glutamine. The enzyme, when extracted from the cells, was shown to be stable (actually stabilized) during 48-hour exposure at 37° C to large volumes of the glutamyl transferase reaction medium, which contains glutamine. Observations of this kind indicate that the manner in which specific enzymes participate in protein turnover may be an important factor in determining their activity levels, not only in cultured cells but also in cell populations of the body that exhibit small net changes in cell number or total protein content.

The use of acetone at room temperature for fixing cells before enzyme assay will be regarded as a brutal procedure by many. In each instance where this procedure has been adopted, the activities found after acetone fixation have equaled or significantly exceeded those found after simple rinsing, air-drying, fixation with cold acetone, or after extraction of the enzyme by sonic vibration or other treatments. Some of the enzymes which withstand the treatment are acid phosphatase, alkaline phosphatase, β-glucuronidase, γ-glutamyl transferase, and ornithine transcarbamylase. The benefits of monolayers for enzyme assay can be gained without the tedium of washing, sterilizing, and immobilizing coverslips in petri dishes, so that most of the work with glucuronidase was executed by assaying the enzyme activities directly on monolayers in dishes lacking coverslips. However, coverslip cultures were also used as planchets to determine radioactivity in the cells, for microscopic determinations of cell number, and for cytological study as accompaniments of enzyme studies.

Our painstaking work to detect small interstrain differences in enzyme activity has led us to perform a great deal of labor and may appear as a prime example of nit-picking. The increase in enzyme activity in a trisomic cell might be as small as 1.5-fold, not forgetting that the extra chromosome might cause vaster increases or decreases in activity. The difference between cells homozygous and heterozygous for genetic determinants of normal enzyme activity might be only twofold [(*11*) for example]. Such differences are small and their demonstration is technically demanding. Once reliably established they could play an important part in the study of somatic cell genetics.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD)—THREE PROBLEMS IN REGULATION

Our second interest in gene dosage effects concerns the most obvious candidate for such studies—the X chromosome. Not only do normal males and females differ in having 1 and 2 X's, respectively, but humans with 3, 4 and, even 5 X chromosomes per cell occur. The last 3 types of individuals deviate from the norm, it is true, but their deviations are far less extreme than those accompanying the 3 autosomal trisomies just discussed. This is presumably due to what must now be considered a fact: The genes on only 1 X chromosome are phenotypically expressed in individual somatic cells. This statement needs qualification to be compatible with the obvious differences that distinguish XO from XX individuals, for instance. Nevertheless, almost all the evidence indicates that there is but 1 "active" (*12*) X chromosome per diploid cell, all others of the same cell being "inactivated" (*13*). Thus, the chromosome most favorable for the study of gene dosage effects has evolved in a manner designed to cancel the effect. This becomes clear when we consider that the determinants for G6PD in man are on the X chromo-

some (*14–16*), and the enzyme levels in erythrocytes do not vary with the number of X chromosomes of the individuals studied.

The first evidence for the single-active-X notion actually was derived by examining qualitative rather than purely quantitative differences. Female mice that were heterozygous for various sex-linked determinants of coat color did not have pelts of uniform color. Rather, their coats were patchworks of areas expressing the color phenotype of one allele or the other, but not both. The fact that cells of similar phenotype occurred in large patches suggested another important aspect of the regulation of X-chromosome behavior: that the initial inactivation events occurred early in embryonic life. Either X could be involved in a given cell, but once inactivated, a chromosome produced replicas that were also inactive in subsequent generations of somatic cells, *i.e.*, the differentiation between X's is persistent.

These notions have a striking cytological corollary. All but 1 of the X chromosomes of a diploid, somatic cell form a sex chromatin body (*17*). Each such body is an X chromosome which is condensed during interphase, and it has been generally assumed that the observed condensation is, itself, related to the failure of phenotypic expression of genes on the inactive X's. This has not been proved. The situation can be summarized in 3 statements:

1) The genes on only 1 X chromosome per diploid set are phenotypically expressed in diploid cells.

2) "Active" X chromosomes produce replicas that are active, while the replicas of "inactive" chromosomes are, likewise, inactive in successive cell generations.

3) "Inactive" X chromosomes form sex chromatin bodies and their replicas do so.

Cultivated human cells are ideal material for demostrating the truth of these statements for a variety of reasons. More than 90 percent of the cells in cultures of the fusiform, diploid cells derived from the skin of females do form a sex chromatin body under appropriate conditions and the proportion of cells that could do so is probably closer to 100 percent. This aspect of the differentiation between the X's clearly persists in the culture environment, nicely enough to provide evidence favoring statement 3 (*18*).

In order to assess the accuracy of statements 1 and 2 one requires cells that are heterozygous for alleles of an X-linked determinant, where both alleles are phenotypically expressed in culture. Single cells of this type, or their clonal progenies, should then express the phenotype of only one allele or the other if the statements are true. The requirements for such analysis are well satisfied by the mutations that alter the electrophoretic mobility of G6PD. These are sex-linked (*15*, *16*) and heterozygous females, to serve as donors of cells for culture, are readily detected by subjecting the G6PD of erythrocytes to starch gel electrophoresis, selecting females where two variants of the enzyme are found. We have shown (*19*) that cells cultured from the skin of an individual produce the same

electrophoretic variants of G6PD as are found in the red cells of the same individual. Figure 1 depicts a starch gel containing the two commonest species of G6PD, as found in cultured cells. Slot 1 contains the rapidly migrating variant A, while slot 5 contains the more slowly migrating, "wild-type" variant B. Slots 2, 3, and 4 contain extracts of cells from 3 females that produce both variants. The hypothesis of the single-active-X predict that clonal isolates from such cell populations should produce only A or B, not both. This has just been demonstrated in elegant fashion (*20*).

The G6PD's of the cell populations depicted in figure 1 reflect the operation of the control scheme. Variants A and B occur in approximately equal amounts in the cells of slots 3 and 4 but A is the predominant component in the cells of slot 2. Variant B predominated in cells cultivated from other heterozygous females. The wide range of variability in relative amounts of A and B among heterozygotes is predicted by the hypothesis, for the X-inactivation event is said to occur at random in the populations of embryonic cells. If this is true then different cell populations from the same heterozygous female should also occasionally manifest large differences in the relative amounts of G6PD variants. Comparisons of the G6PD's in populations of red cells and cells cultivated from skin of the same individual display this variability (*19*).

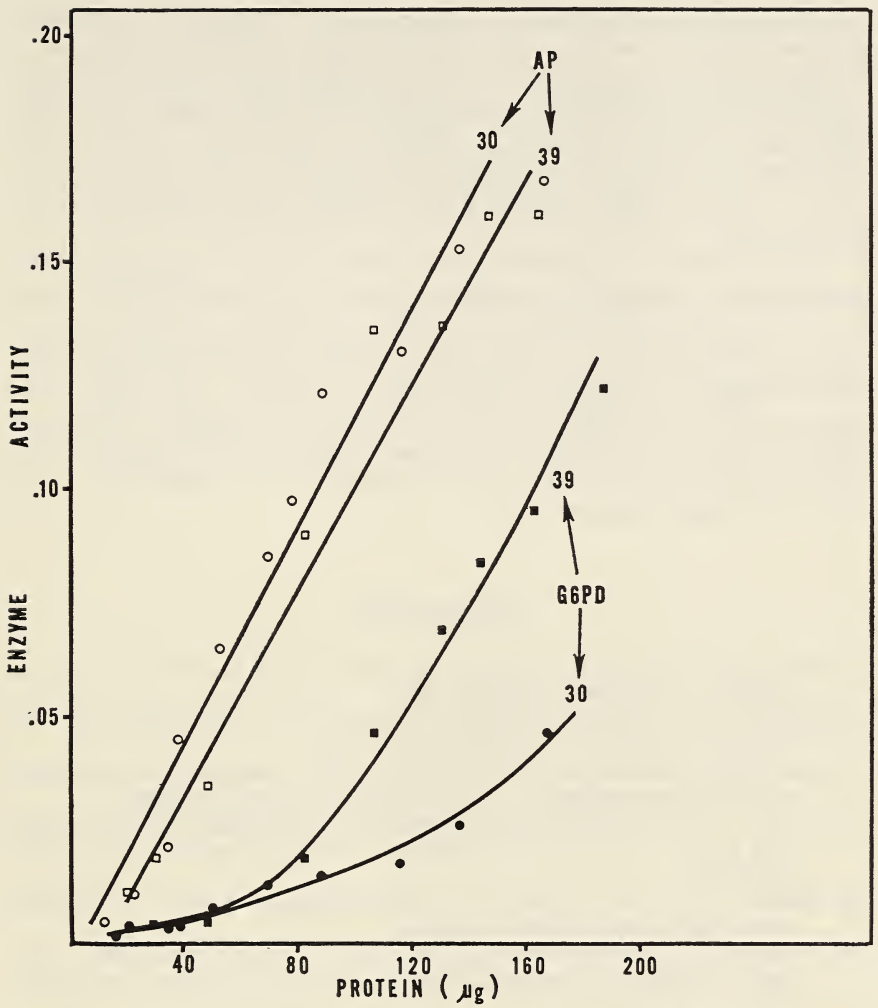
The operation of the single-active-X scheme suggests an interesting observation, concerning the origin of malignancies, that might be made with females heterozygous for G6PD determinants. If the population of malignant cells is the progeny of a single stem cell, as proposed in some theories, then only one variant of the enzyme should be found. If the population is derived from more than one cell or if the influences causing malignancy are transmissible from cell to cell then both varieties of the enzyme might be found. For the population might then consist of a mixture of cells differing with regard to the X that was "active." The assumption inherent in this proposal is that the differentiation between the X's is maintained in the malignant cells. Females of the proper sort for such a study should not be too difficult to locate among American Negroes.

The cultivated cells have proved to be suitable for a complete demonstration of the single-active-X scheme. They will undoubtedly prove themselves useful in future analysis of the underlying basis for the differentiation between the X's. They seem to be well suited to the analysis of two other aspects of control over levels of G6PD activity. The first of these is indicated by curve B of text-figure 1. It was derived by making repeated determinations of G6PD activity in sonically disrupted cells during growth of the cultures. The specific activity of the enzyme varies during growth, increasing as the population density increases. This presents a question about the environmental factors regulating the production of G6PD. The increase curve for G6PD resembles that for β-glucuronidase in a general way, but differs from it in failing to show an increase in activity when net changes in total cell protein cease in high density

populations. In fact, terminal decreases in G6PD activity have been observed in some experiments. It is possible that such decreases are related to a third problem in regulation.

The A variant of G6PD is found in two kinds of individuals. Some of these have normal amounts of G6PD activity, while others have a mutant gene that lowers the activity of the enzyme to about one tenth or one twentieth of the normal value for erythrocytes. This shortage of enzyme activity is not as clearly manifest in other cells of the body. The A enzymes from deficient and nondeficient people are indistinguisable from one another and from the "wild-type" B enzyme when parameters of enzyme activity are compared. Kirkman has even shown (*21*) that the low enzyme activity in erythrocytes of deficient individuals is not associated with the presence of an unusually large amount of G6PD-related but enzymatically inactive protein, *i.e.*, the deficiency mutations seem to cause a shortage of enzyme molecules that, so far, cannot be distinguished from those found in individuals with normal amounts of enzyme activity.

A clue to the understanding of what these particular deficiency mutations do may exist in the fact that the enzyme deficiency is most pronounced in mature erythrocytes, less so in immature erythrocytes, and is hardly detectable in other cells of the body. The enzyme activity seems to decay, even in nondeficient individuals and, perhaps, more rapidly in mutants. The decay may involve a breakdown of the enzyme, only partially, if at all, compensated for in the enucleated red cells. Possibly, the deficiency mutations accelerate the rate of breakdown. If so, the effect is specific for the A variant of the enzyme. The implication here is that the deficiency mutations have not occurred in the determinants of enzyme structure. Alternatively, the mutations might have occurred in such determinants. The alteration in protein structure would then be of a sort undetectable by study of the parameters of enzymatic activity or electrophoretic mobility but would foster unusually rapid breakdown by normal agencies operating at normal intensity. Young erythrocytes might then be expected to have more enzyme activity than older ones since breakdown would have proceeded for less time. Nucleated cells might have near normal amounts of enzyme because losses through breakdown might be restored by resynthesis under the influence of the nucleus. But we believe the enzyme deficiency is detectable in cells cultured from skin. Text-figure 3 depicts a comparison of cells cultivated from subjects with normal activity and electrophoretic variant B with cells from an American Negro female homozygous for determinants of enzyme deficiency and variant A. Repeated determinations of enzyme activity made during growth revealed that the mutant and control cells did not differ significantly until the cultures exceeded one-third to one-half maximum density. Most of the G6PD is formed after this density is exceeded and, in both cases, the mutant strain attained a specific activity that was about one half that of the control. The acid phosphatase activities of the two strains did not differ significantly, which indicated that the differences in G6PD activity were specific although much smaller



TEXT-FIGURE 3.—Appearance of acid phosphatase (AP) and glucose-6-phosphate dehydrogenase (G6PD) activities during growth of cells cultured from human skin. Strain 30 was derived from an American Negro woman who had a deficiency of erythrocytic G6PD activity and produced only electrophoretic variant A in both red cells and cells cultured from skin. Strain 39 was derived from an individual producing only variant B, with normal G6PD activity. The assay procedures are described in the legend of text-figure 1. Values for enzyme activity and protein pertain to populations of cells on coverslips 18 mm in diameter. Values for G6PD are the optical density change per minute at 340 mμ. The acid phosphatase activities were lower than usual but followed the usual pattern (*see* legend of text-fig. 1).

than those found in comparisons made with erythrocytes. A second comparison made with other mutant and control strains gave the same result. It should be possible to study the decay of enzyme activity in cell populations that have become static because they have attained

maximum density or because of some defined nutritional deprivation. Such populations are not truly static, however, since their proteins are in a dynamic state, and resynthesis might restore losses of enzyme activity occurring through breakdown, minimizing interstrain differences. Such differences might be maximized by interfering with protein synthesis with agents such as puromycin or amino acid analogues. Experiments of this sort are hardly feasible with red cells, but seem to be just right for cultivated cells.

This introduces my final comment, which will be recognized as the expression of a personal bias concerning the best types of cells to use for combined genetic and biochemical study. If suffices to note that, in each case, the cells we used were derived from humans with genetic aberrations, either aneuploid or mutational, that were well defined. This permitted a firm relation to be drawn between phenotypic variations and special genetic properties, when such variations existed. The drawing of such relations is basic to *in vitro* genetic analysis and the diploid cells would seem to be ideal for the purpose.

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PLATES

PLATE 18

FIGURE 1.—Electrophoretic variants of glucose-6-phosphate dehydrogenase (G6PD) in cells cultured from human skin. Donors were American Negro women. Slot 1: homozygous for variant A. Donor had a pronounced deficiency of erythrocytic enzyme activity. Slot 5: homozygous for variant B. Donor had a normal level of erythrocytic enzyme activity. Slots 3, 4, and 5: heterozygous for variants A and B. Donors had intermediate levels of erythrocytic G6PD activity. *See* (*19*) for details of procedure.

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ENZYMES OF ARGININE METABOLISM IN CELL CULTURE: STUDIES ON ENZYME INDUCTION AND REPRESSION [1]

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The control of metabolic processes by alterations in contents of critical enzymes by the mechanisms of enzyme induction and repression has been well established in various microorganisms (*1*). Demonstrations of similar control mechanisms in intact mammalian tissues have been hindered by the inherent complexities of the intact animal. The isolated mammalian cell grown in continuous culture, on the other hand, affords a far more simplified system for studies of enzyme induction and repression in mammalian tissues. The studies presented are on the series of enzymes, shown in text-figure 1, which are involved with the synthesis of arginine from citrulline, *i.e.*, argininosuccinate synthetase and argininosuccinase, and with the degradation of arginine, *i.e.*, arginase. The two enzymes involved in arginine biosynthesis are affected by the concentration of arginine in the growth medium, so that when the concentration of arginine in the medium limits growth, the levels of argininosuccinate synthetase and argininosuccinase are from 2-fold to 15-fold greater than when the cells are grown in excess arginine. This phenomenon is similar to arginine repression as demonstrated in *Escherichia coli* (*2*) and *Bacillus subtilis* (*3*) and indicates some similarities between control of arginine biosynthesis in microorganisms and higher animals.

The level of arginase has a direct relation to the concentration of arginine in the medium, that is, it is controlled by a substrate inductive effect. A similar effect of arginine has been noted by Klein (*4*). Further, the addition of manganese to the medium profoundly increases arginase activity of the cells. The mechanisms of these increases in enzyme activity were investigated with a specific antiarginase antibody, and were found to be related not only to the rate of *de novo* synthesis of the arginase, but also to the rate of its turnover, *i.e.*, breakdown.

Although the following observation is unrelated to enzyme induction and repression in mammalian cells, it should be emphasized that in studies of metabolic processes in cell cultures the cell lines used must be rigidly free of contamination by pleuropneumonia-like organisms (PPLO). The

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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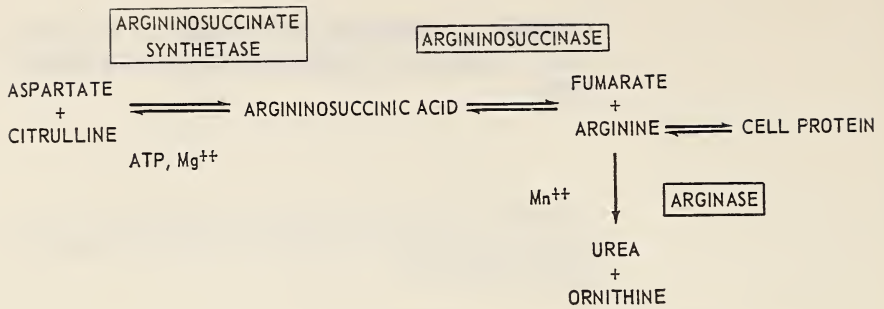


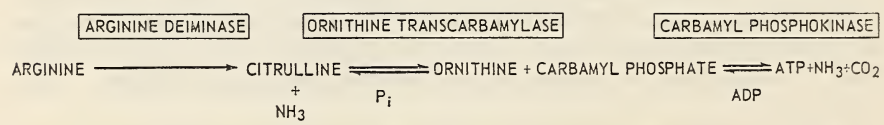
TEXT-FIGURE 1.—Arginine metabolism in cultured mammalian cells.

frequent, unapparent contamination of cell cultures by these organisms may lead to false conclusions. This is particularly true with studies of arginine metabolism since, in the presence of PPLO, arginine is rapidly degraded to ornithine by the pathway shown in text-figure 2. This pathway is present in all PPLO strains isolated from cell culture, and is absent in all PPLO-free cultures (*5*).

MATERIALS AND METHODS

Cell cultures.—PPLO-free lines of HeLa-S_3, HeLa, KB, and L-929 were grown in suspension culture in Eagle's spinner medium (*6*) with 5 percent (HeLa-S_3 and L) or 10 percent (HeLa and KB) horse serum filtered through Sephadex G-50 before use. Cells were harvested and resuspended in medium containing specific concentrations of arginine, citrulline, etc., as described elsewhere (*7*). Growth of the cells was determined by either cell counts with a hemocytometer or by protein determinations on cells washed with serum-free medium (*8*). Cell populations were routinely maintained from 2 to 6 $\times$ 10^5 cells per ml.

Enzyme assays.—The details of preparation of cells and assays for argininosuccinate synthetase, argininosuccinase, and arginase were presented elsewhere (*7*). The assays of argininosuccinate synthetase and argininosuccinase utilized C^{14}-labeled amino acid substrates, *i.e.*, ureido-C^{14}-L-citrulline, obtained from the New England Nuclear Corporation (argininosuccinate synthetase), and guanido-C^{14}-L-argininosuccinic acid (argininosuccinase), synthesized according to Ratner (*9*). The product of the immediate reaction is converted to urea by the addition of excess amounts of ancillary enzymes according to the pathway indicated in



TEXT-FIGURE 2.—Arginine breakdown in pleuropneumonia-like organisms-contaminated cell cultures.

text-figure 1. The urea, labeled with C^{14}, was decomposed by urease, and the liberated $C^{14}O_2$ was collected and counted. Results of all assays are linear with respect to time and tissue concentration under the existing conditions.

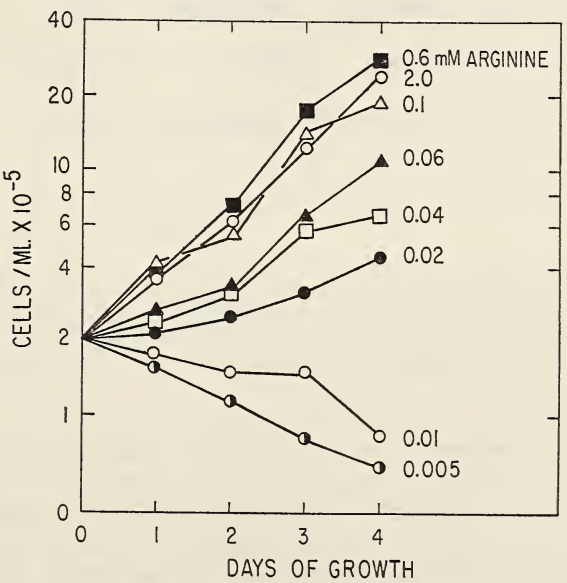
Immunologic studies.—The details of the preparation of the anti-arginase antibody from rabbits, with the use of purified rat liver arginase and the preparation of HeLa-S_3 extracts for precipitin reactions have been described (*10*).

RESULTS

Repression of Argininosuccinate Synthetase and Argininosuccinase

Repression-derepression, *i.e.*, the ability to alter the rate of synthesis of a series of biosynthetic enzymes by varying the availability or concentration of the end product, has classically been demonstrated to its fullest extent in microorganisms when the specific end product was present in an amount small enough to limit growth (*11*). The repression effect of arginine on levels of the two enzymes involved in its biosynthesis in mammalian cells can also be demonstrated only when the cells are grown under conditions in which arginine limits growth.

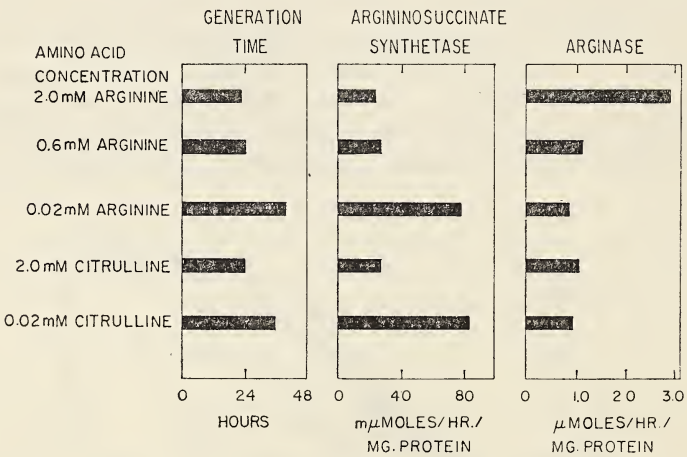
Text-figure 3 indicates the range of arginine concentrations which limited growth in HeLa-S_3. Between the arginine concentrations of 0.1 mM and 0.02 mM, there was a direct relationship between growth rate and arginine concentration. Increasing the concentration of arginine



TEXT-FIGURE 3.—Effect of varying arginine concentrations of medium on HeLa-S_3 cells grown for 4 days. Medium was replaced daily without removal of any cells.

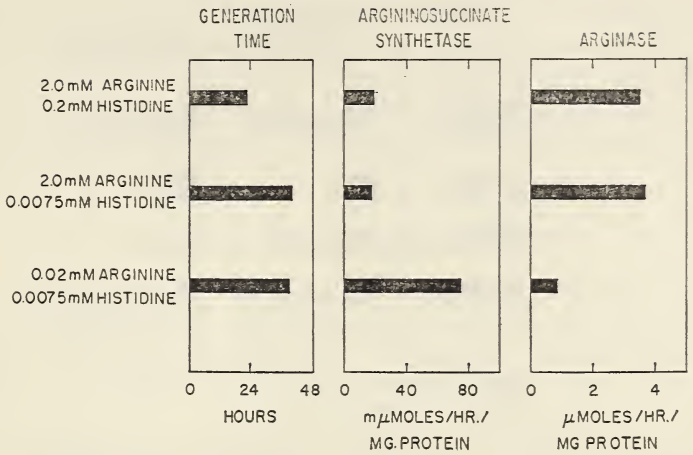
above 0.1 mM did not further increase growth rates. Decreasing the concentration of arginine below 0.02 mM did not further decrease growth but resulted in cell lysis and death. These data are similar to those of Eagle *et al.* (*12*) with lysine, threonine, and valine.

Text-figure 4 shows the effects in suspension cultures of HeLa-S_3 of varying the concentration of arginine and citrulline in the medium on levels of argininosuccinate synthetase, representing an enzyme of the biosynthetic pathway, and of arginase, as the control enzyme. The level of argininosuccinate synthetase activity was increased threefold to fourfold under conditions in which arginine, at a concentration of 0.02 mM, limited growth. High concentrations of citrulline (2.0 mM) produced maximal growth and had no effect in increasing argininosuccinate synthetase activity. Concentrations of citrulline that supported limited growth also resulted in increased levels of argininosuccinate synthetase, but no higher than those occurring with limiting arginine concentrations. The effects of arginine on the levels of arginase are the opposite of those on argininosuccinate synthetase, the level of arginase increasing with the arginine concentration. High concentrations of citrulline in the medium, however, did not increase levels of arginase activity.



TEXT-FIGURE 4.—Effect of varying concentrations of arginine and citrulline on growth and levels of argininosuccinate synthetase and arginase of HeLa-S_3 cells. Cells were grown for 4 days in medium containing the indicated concentrations of arginine or citrulline before enzyme assays. Medium was replaced every 48 hours.

Text-figure 5 presents the results of an experiment showing that changes in enzyme levels were not simply due to retardation of growth. In this experiment growth was limited by decreasing the concentration of histidine in the medium, but this limitation by histidine in the presence of high arginine concentrations did not alter enzyme levels. Only when the arginine concentration was also diminished was the level of argininosuccinate synthetase increased.

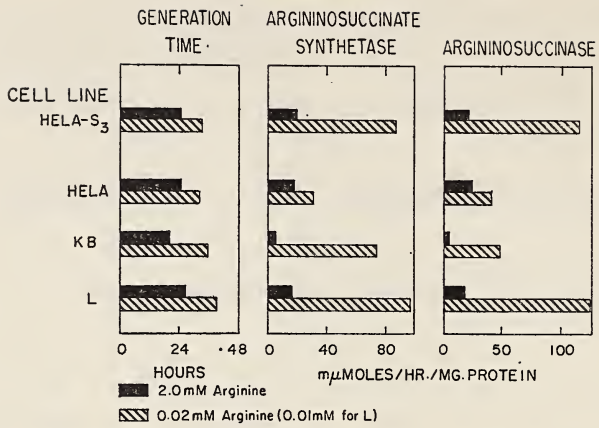


TEXT-FIGURE 5.—Effect of growth limitation by histidine on argininosuccinate synthetase and arginase of HeLa-S_3 cells. Cells were grown for 4 days in medium containing indicated concentrations of arginine and histidine before harvest and enzyme assay. Medium was replaced every 48 hours.

Text-figure 6 shows the results of a series of cell lines studied for the existence of the arginine repression effect. In addition to HeLa-S_3, lines of HeLa, KB, and L-929 were examined for argininosuccinate synthetase and argininosuccinase activities under conditions of excess arginine (2.0 mM) and limiting (0.02 mM or 0.01 mM for the L-929 cell line) arginine. All cell lines showed an effect that varied in extent. In the HeLa line, the repression effect was least striking, whereas in KB and L-929 cells the differences between cells grown in high or low arginine were up 10-fold to 15-fold. There were comparable changes in the levels of both enzymes involved in arginine biosynthesis, that is, they were affected in a coordinated manner. As with HeLa-S_3 cells (text-fig. 4), the substitution of citrulline for arginine gave no higher enzyme levels than limiting concentrations of arginine, which indicated no "inductive" effect of the citrulline.

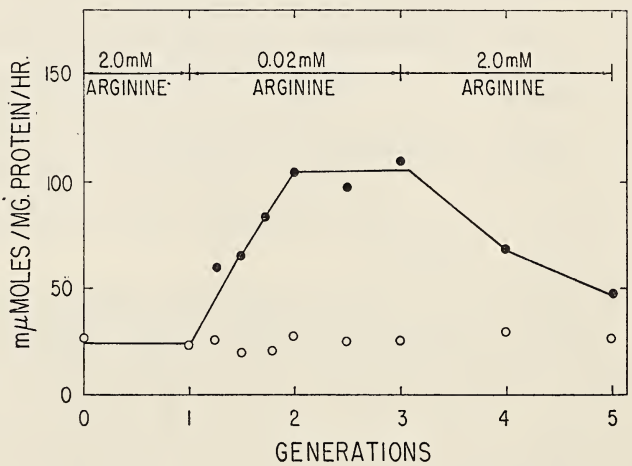
Many experiments have failed to demonstrate the presence of activators or inhibitors of argininosuccinate synthetase, argininosuccinase, or arginase activity in extracts of the cells, or a direct effect of arginine on the enzyme activities under the assay conditions used. Studies of apparent Michaelis constants and *p*H optima of argininosuccinate synthetase and argininosuccinase have also failed to indicate any qualitative differences in the enzyme activities.

Text-figure 7 shows the results of a typical experiment of the time course of increase in argininosuccinase produced when cells are changed from a medium containing excess arginine to one containing a limiting concentration. The increase in argininosuccinase activity was completed within one generation of growth. With change back to a medium containing excess arginine, the enzyme activity was stable. In other experi-



TEXT-FIGURE 6.—Effect of varying arginine concentrations of medium on argininosuccinate synthetase and argininosuccinase. PPLO-free cell lines were grown 4 days in indicated concentrations of arginine before harvest and enzyme assays.

ments in which the cells were maintained in limiting arginine concentrations up to 10 days, a similar time course of increase, decrease, and extent of change in argininosuccinase activity was found. This result excludes the possibility that the arginine effect was due to a selection of cells containing a higher level of the enzyme rather than a change within the cells.

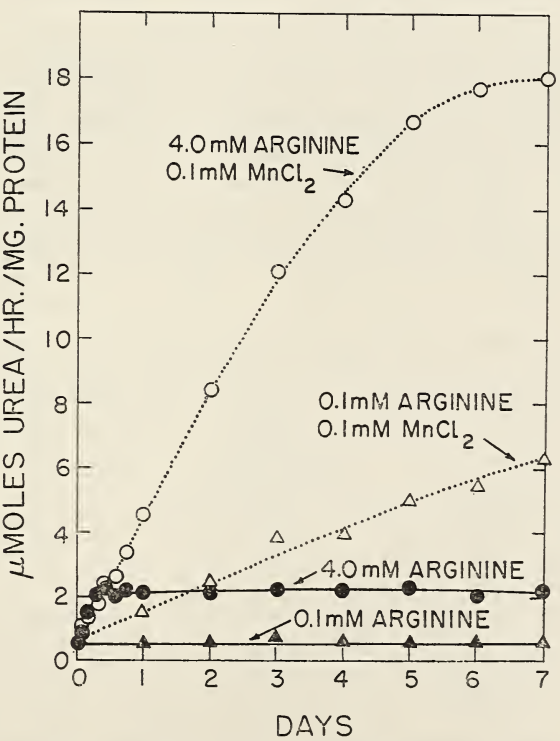


TEXT-FIGURE 7.—Time course of change in argininosuccinase activity of HeLa-S_3 cells produced when the concentration of arginine in the medium was altered. Cells maintained in 2.0 mM L-arginine were transferred to medium containing 0.02 mM arginine (●—●) and assayed daily for argininosuccinase activity for a total of two generations of growth. Medium was replaced daily. Cell concentrations were maintained from 2 to 4 × 10^5 cells per ml. Cells maintained in 2.0 mM L-arginine (○—○) were assayed daily for control.

No ornithine transcarbamylase activity (ornithine + carbamyl phosphate→citrulline) was demonstrated under any growth conditions. This finding is in keeping with the fact that ornithine is unable to substitute for the arginine requirement of cultured cells (*6*), and indicates that the absence of ornithine transcarbamylase is a genetic defect and not simply associated with repression.

Induction of Arginase in HeLa-S_3

Two separate factors involved in the control of levels of arginase activity were found. In addition to the effect of arginine previously described (text-figs. 4 and 5), there was a more profound effect when manganese was added to the medium, and a lesser effect when cobalt and nickel were added. The time course of these separate and additive effects of arginine and manganese is shown in text-figure 8. In these experiments HeLa-S_3 cells originally maintained in 0.1 mM arginine were transferred to medium containing 0.1 mM arginine, 0.1 mM arginine plus 0.1 mM $MnCl_2$, 4.0 mM arginine, or 4.0 mM arginine plus 0.1 mM $MnCl_2$.



TEXT-FIGURE 8.—Time course of increase in arginase activity produced by addition of high arginine and/or $MnCl_2$ to medium. HeLa-S_3 cells maintained in 0.1 mM L-arginine were placed in medium containing indicated concentrations of arginine and manganese. Medium was replaced daily. Aliquots were removed daily and the cells assayed for arginase activity.

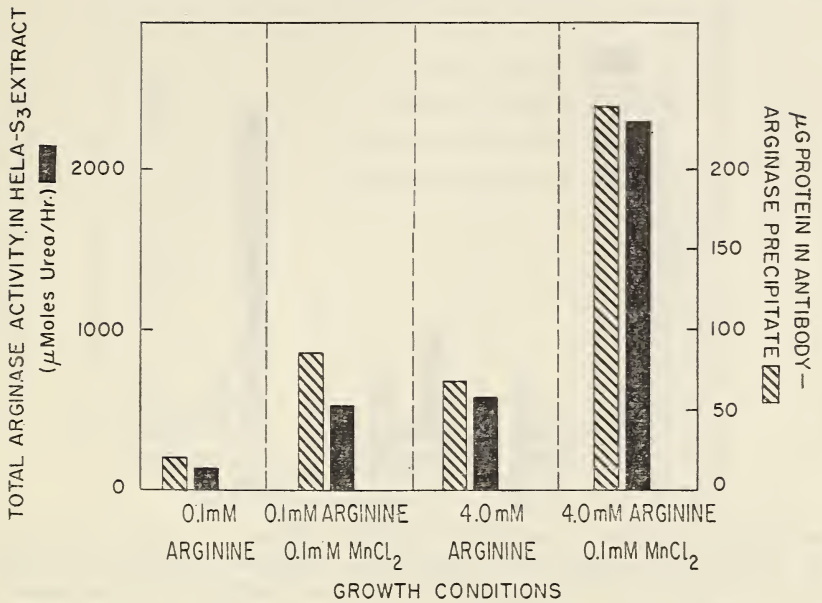
Samples were assayed daily for arginase activity. Cell growth rates were similar under all conditions of growth. The arginine effect resulted in a fourfold to fivefold increase in specific enzyme activity (μmoles of urea formed/hour/mg protein). This increase was completed within 12 hours after the change in medium. The manganese effect was strikingly different in time course, with linear increases in enzyme activity for up to 3 to 5 generations of growth. The time course of increases in arginase activity produced by addition of manganese to the medium occurred at two different rates, being 2 to 3 times as rapid in the presence of 4.0 mM arginine as with 0.1 mM arginine.

The changes in enzyme activities indicated in text-figure 8 could result from a number of mechanisms. The presence of activators or inhibitors must always be considered. These were ruled out by various experiments involving the mixing of aliquots of extracts from cells having widely differing specific enzyme activities. Furthermore, the arginase assays were routinely performed under conditions of maximal activation of arginase by manganese, and hence the manganese effect cannot be readily ascribed to a simple activation of the enzyme.

A number of experiments were performed which indicated that the changes in enzyme levels were due to changes in content of enzyme protein, and that increases in content of enzyme protein occurred by *de novo* protein synthesis. For most of these studies it was necessary to isolate specifically the arginase protein in high purity and high yield. Conventional enzyme purification procedures were totally inadequate because of the small amount of material. The use of an antibody precipitating only arginase protein from cell extracts has proved of inestimable value in elucidating the mechanisms for increased arginase activities produced by arginine and manganese. Figure 1 shows an Ouchterlony gel diffusion plate (*13*) of a rabbit antiarginase antiserum, a highly purified, homogeneous arginase purified from rat liver (*14*), the original antigen (well 2), and two extracts of HeLa-S_3 with differing specific enzyme activities (wells 1 and 3). The antiserum was placed in the center well. It is evident that similar precipitin reactions have taken place irrespective of the growth conditions of the cells, and that there is virtually complete cross reactivity with this antibody preparation.

It has been possible to demonstrate that the differences in enzyme activity resulted from differing contents of specific enzyme protein rather than the presence of activators, inhibitors, or alterations in the kinetic properties of the arginase protein. Text-figure 9 illustrates a comparison between the measurable enzyme activity as altered by growth for 24 hours in various combinations of arginine and manganese and the amount of antiarginase precipitate. It is apparent that the amount of protein precipitated and the measurable enzyme activity are comparable.

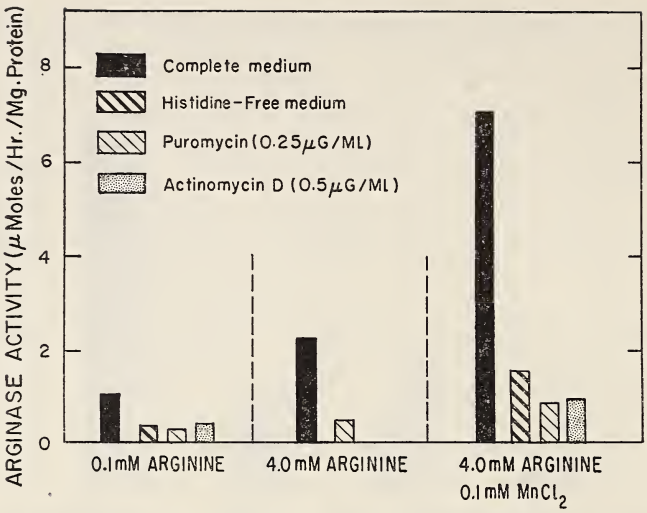
The increased content of arginase protein produced by an increase of arginine concentration in the medium, or the addition of manganese to the medium, could arise from either *de novo* synthesis from constituent amino acids, or by conversion of a preformed precursor to an enzymatically



TEXT-FIGURE 9.—Comparison of arginase activity and protein precipitable by anti-arginase in HeLa-S_3 extracts. Cells maintained in 0.1 mM arginine were grown 24 hours in the media. Extracts of cells from each of the flasks (2000 ml of culture fluid) were assayed for arginase activity, after which aliquots were added to the antiarginase and the amount of precipitated protein was compared with the measured enzyme activity. The values for precipitated protein were corrected for a small amount of precipitate present in extracts added to nonimmunized serum. The enzyme activity was quantitatively recovered from the precipitated protein. No enzyme activity was present in the precipitate of the control serum and HeLa-S_3 extract.

and immunologically active protein. Indirect evidence for *de novo* synthesis was obtained from studies in which various means of inhibiting protein synthesis during the "induction" period were used (text-fig. 10). The addition of either puromycin (*15*) or actinomycin D (*16*), or the removal of histidine from the medium abolished the increases in arginase produced by either increased arginine concentrations or the addition of manganese to the medium. Two additional findings were: In the presence of inhibitors of protein synthesis, when the cells were maintained in the basal medium containing 0.1 mM arginine, the specific activity of arginase was consistently diminished 30 to 40 percent of control levels. Furthermore, when manganese was added to the medium this decline in specific activity was prevented. The possible significance of these findings will be discussed later.

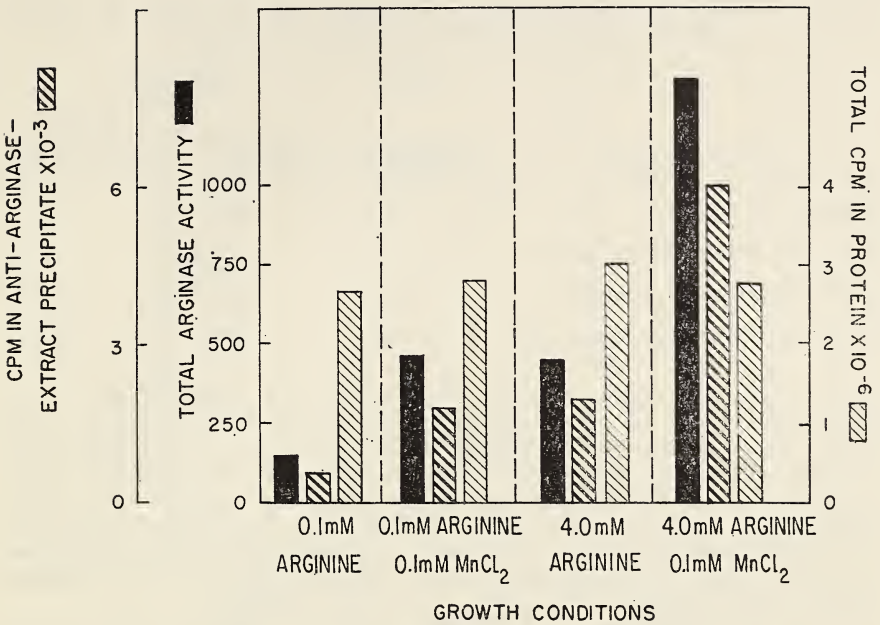
A more definitive demonstration of the *de novo* synthesis of arginase was obtained with the use of amino acid-labeling experiments, isolating the arginase protein by means of the antiarginase antisera. Text-figure 11 shows the results of an experiment in which cells grown in 0.1 mM arginine were changed to media containing the indicated concentrations of arginine



TEXT-FIGURE 10.—Effect of inhibitors of protein synthesis on arginase "induction" by arginine and manganese (24 hours' growth of HeLa-S_3 maintained previously in 0.1 mM arginine). Cells were grown in 0.1 mM L-arginine and then placed in media containing the concentrations of arginine and manganese, plus the additions or deletions indicated. Cells were grown an additional 24 hours, then harvested and assayed for arginase activity.

and manganese and grown in the presence of C^{14}-L-histidine for 24 hours. The cells were then harvested and the counts incorporated into total protein and protein precipitable with the antiarginase antiserum were compared to enzyme activity. The amount of label incorporated into total cell protein was essentially similar under the four conditions. The counts in the antiarginase precipitate, on the other hand, were proportional to the enzyme activity present in the extracts.

In another experiment cells were grown for five generations in medium with 0.1 mM arginine, containing S^{35}-L-cystine, thereby producing a culture with complete and uniform labeling of the protein. At zero time a portion of the cells was harvested, the remaining cells were centrifuged and replaced with medium containing S^{32}-L-cystine, and either 0.1 mM arginine or 4.0 mM arginine plus 0.1 mM manganese, and grown an additional 24 hours before harvest. The results of this experiment are shown in table 1. There was a sixfold increase in arginase activity produced in cells grown in medium containing 4.0 mM arginine plus 0.1 mM manganese. If a preformed, and necessarily labeled, precursor accounted for this increase in enzyme protein, then there should have been a comparable increase in counts found in the antiarginase precipitate. It is evident that there was no increase in counts over those present at the time the labeled cystine was removed from the medium. This experiment indicates conclusively that the precursor hypothesis is untenable and, together with the previous experiment, rules out the possibility that the increased arginine and manganese caused a molecular modification in cellular



TEXT-FIGURE 11.—Incorporation of C^{14}-histidine into total protein and arginase during arginase "induction" by arginine and manganese. Cells grown in 0.1 mM L-arginine were placed in the specified media containing 20 μc C^{14}-L-histidine, total volume of culture 2000 ml for each medium, and grown an additional 24 hours. Cells were harvested and separated into protein precipitable by antiarginase and total cell protein.

protein that resulted in an active protein, both enzymatically and immunologically.

Both the effect of arginine and the effect of manganese can be considered as examples of enzyme induction, that is, they cause an increase in enzyme protein. However, the time course studies shown in text-figure 8 indicate that the effects of arginine and manganese were not similar. Enzyme induction has been generally considered to result in an "increased synthesis of enzyme protein" (*17*). In growing bacteria, the accumulation of an enzyme can be accomplished only if the rate of synthesis relative to other cell proteins is increased, since there is no demonstrable protein turnover (*18*). But in mammalian tissues, both in cell culture and the intact animal, protein turnover is a consistent and constant finding (*19, 20*). Thus, the processes determining the level of a given protein may be depicted as follows:

$$\text{Amino acids} \xrightarrow[\text{Synthesis}]{} (\text{Enzyme}) \xrightarrow[\text{Breakdown}]{} \text{Breakdown products (amino acids).}$$

The accumulation of increased amounts of a given enzyme protein may result from increased rates of synthesis and/or decreased rates of breakdown.

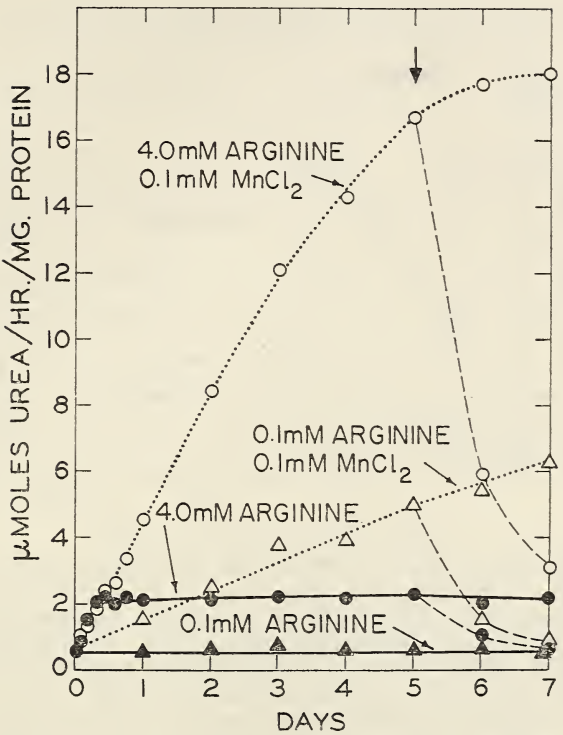
TABLE 1.—Loss of S^{35}-L-cystine label from arginase and total protein of HeLa-S_3 during arginase "induction" by arginine and manganese

Growth conditions	0 Time	24 Hours	
	0.1 mM Arginine	0.1 mM Arginine	4.0 mM Arginine plus 0.1 mM $MnCl_2$
Total cell protein (mg)	168	250	241
Total cpm S^{35}-L-cystine $\times 10^{-5}$	7. 33	5. 35	5. 75
Total arginase (μmoles urea/hr)	126	185	1, 222
Total cpm S^{35}-L-cystine in arginase	1, 020	322	895

HeLa-S_3 cells were grown in medium containing 0.1 mM L-arginine and 0.1 mM S^{35}-L-cystine, specific activity 0.3 mc per mM, for a total of five generations. The cells, in a total volume 4500 ml, were divided into three portions. One portion was harvested immediately, and the remainder was washed and replaced with medium containing 0.1 mM S^{32}-L-cystine plus 4.0 mM arginine, 0.1 mM manganese, or 0.1 mM arginine only, and grown for 26 hours. The cells were harvested, and the counts incorporated into total cellular protein and into protein precipitable by antiarginase were determined. These were compared with total cell protein and total arginase activity in the extracts.

A considerable accumulation of data suggests that arginase in HeLa-S_3 cells has a significant turnover, and hence the rate of breakdown may well affect the given level of the enzyme. Text-figure 12 shows a part of the experiment illustrated in text-figure 8, in which the cells were returned to a medium containing the basal 0.1 mM arginine. It is apparent that the loss of enzyme activity was far more rapid than can be explained by dilution due to continued cell growth with arginase produced at the level characteristic of growth in 0.1 mM arginine. There is further evidence of a rapid turnover in the experiments in which inhibition of protein synthesis produced a rapid decrease in arginase activity (text-fig. 10).

A labeling experiment to demonstrate turnover is illustrated in text-figure 13. Cells were grown in 2.0 mM arginine. At zero time S^{35}-L-cystine was added to each flask and samples were removed at the indicated intervals. One portion of the cells was grown for five generations in the S^{35}-L-cystine to produce a uniformly labeled culture. The results are expressed as percent of maximal labeling obtained after five generations of growth in S^{35}-L-cystine. If no turnover at all occurred after 24 hours of growth (one generation), the samples of cell protein and arginase should have contained 50 percent of the maximal label, as indicated by the "theoretical" curve. However, it is evident that such was not the case. The protein precipitable with the antiarginase antiserum was maximally labeled within 12 hours, which indicates that the entire content of the enzyme was resynthesized and degraded within the 12-hour period. It is clear that arginase turnover occurred at a relatively rapid rate compared to the turnover of total cell protein.

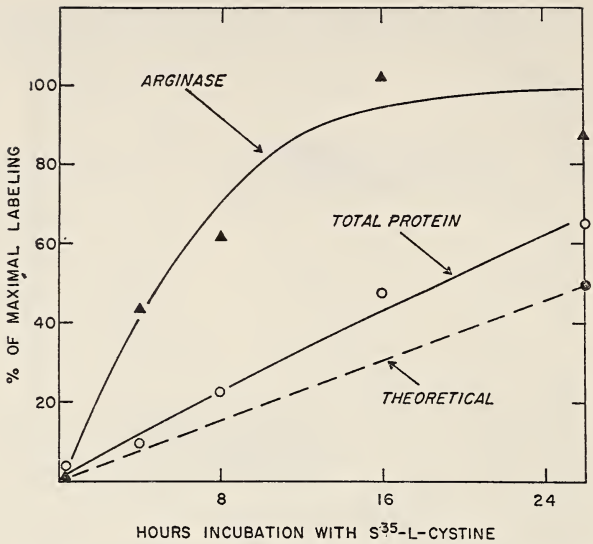


TEXT-FIGURE 12.—Time course of decrease in arginase activity produced by change of HeLa-S_3 to medium containing 0.1 mM arginine. Details of handling of cells are described in text-figure 8. At time indicated by *arrow* a portion of the cells grown in each type of medium was placed in medium containing 0.1 mM L-arginine. Arginase activities were determined daily thereafter for 2 days.

Secretion of arginase by the cells was excluded as the cause of the demonstrated turnover as there was no arginase activity attributable to the cells in the growth medium; arginase of cell lysates incubated in culture medium for 24 hours can be readily detected.

The incorporation of label into total protein was also more than that predicted if no turnover existed. The calculated turnover of total protein based on the results shown in text-figure 13 is 1 percent per hour. This value is similar to that previously reported by Eagle *et al.* (*20*).

It is suggested that the mechanism of manganese effect involves a decrease in the breakdown of the enzyme; *i.e.*, its stabilization. Such a hypothesis would account for the linear increase in specific arginase activity over several generations of growth (text-fig. 8). This hypothesis is supported by the results of the experiment shown in table 2. Cells were grown in 4.0 mM arginine for four generations with S^{35}-L-cystine. At zero time the medium was replaced with medium containing S^{32}-L-cystine and 4.0 mM arginine with and without 0.1 mM $MnCl_2$ and grown for 24 hours before harvest. With no manganese there was a rapid loss of total



TEXT-FIGURE 13.—Incorporation of S^{35}-L-cystine into cell protein and arginase of HeLa-S_3. HeLa-S_3 were grown, in 5 flasks, in 2.0 mM L-arginine and 0.1 mM S^{32}-L-cystine to a final volume of 10 liters (2.5×10^5 cells/ml). At zero time S^{35}-L-cystine was added to a final specific activity of 0.42 mc per mM. At specified intervals 1500 ml of the culture was harvested and counts incorporated into total cell protein and arginase were determined. A portion of the culture was grown for five generations in S^{35}-L-cystine medium to produce complete labeling of all cell protein. The values obtained are expressed as the percent of specific activity (cpm/mg for total cell protein, and cpm/100 units of arginase for arginase) of that obtained with the maximally labeled cells.

counts precipitable with the antiarginase antibody, which again indicates the rapid turnover of the enzyme. If arginase was stabilized by the presence of manganese, there should have been less loss of label than in its absence. The results indicate that there is, indeed, little loss of label from arginase when manganese is added to the medium and that stabilization of arginase has occurred.

At the present time there is not much available information on the mechanism of the arginine effect. However, the different rates of accumulation of arginase produced when cells are grown in the presence of manganese at two different concentrations of arginine in the medium (text-fig. 8) can best be explained by the assumption that arginine affects the rate of enzyme synthesis by acting as an inducer in the classical bacterial meaning of the concept. However, a separate type of stabilization effect has not been excluded. Further studies on the mechanisms of the separate arginine and manganese effects are in progress.

Conversion of Arginine to Urea and Ornithine by Arginase in the Intact Cell in Culture

The extent to which arginine is converted to urea and ornithine, *i.e.*, by arginase, is dependent as much on the concentration of arginine in the

TABLE 2.—Loss of S^{35}-L-cystine label from arginase and total protein of HeLa-S_3 cells during arginase "induction" by manganese

Growth conditions	0 Time	24 Hours	
	4.0 mM Arginine	4.0 mM Arginine	4.0 mM Arginine plus 0.1 mM $MnCl_2$
Cell protein (mg)	131	195	205
Total cpm S^{35}-L-cystine $\times 10^{-5}$	5. 65	4. 10	4. 22
Total arginase (μmoles urea/hr)	310	462	1, 068
Total cpm S^{35}-L-cystine in arginase	2, 540	1, 012	2, 020

HeLa-S_3 were grown in medium containing 4.0 mM L-arginine and S^{35}-L-cystine as indicated in table 1. Conditions of growth and experimental procedures were similar to those given in table 1, except that the cells were grown in medium containing 4.0 mM L-arginine at all times.

medium as on the content of the enzyme arginase. The data in table 3 show the extent (percent) of conversion of arginine to urea, determined in growing cultures of HeLa-S_3 as a function of the arginine concentration. The amount of arginine converted to ornithine and urea, by the arginase reaction, was essentially negligible at concentrations of arginine generally used in culture media, *i.e.*, 0.1 mM. Significant arginine conversion occurred only when the concentration of arginine in the medium was increased to 10 mM, at which 15 percent of the arginine was converted, as compared to 0.01 percent at 0.1 mM arginine. This large difference in extent of conversion was not associated with a comparable increase in enzyme specific activity, as measured under optimal conditions of arginine concentration (250 mM at *p*H 9.5). Thus the increase in extent to which arginine is metabolized by the arginase reaction is probably because the concentration of arginine required for half-saturation of the arginase in cell extracts, *i.e.*, Michaelis constant, is of the order of 10 to 15 mM arginine (*10*). This point is emphasized because it is often forgotten that the substrate concentration may be equally, or more, important in the determination of the over-all rate of a physiological reaction—such appears to be the case with arginine breakdown by arginase in cell culture.

DISCUSSION

We have demonstrated the presence of argininosuccinate synthetase and argininosuccinase activities in several lines of mammalian cells, thereby accounting for the ability of these cells to utilize citrulline instead of arginine for growth. It has further been demonstrated that the levels of these two enzymes are not constant, but vary inversely with the concentration of arginine in the medium. Examples of enzyme changes

TABLE 3.—Dependence of the conversion of arginine to ornithine and urea on concentrations of arginine in the growth medium

Arginine in medium (mM)	Total amount of arginase in extracts* (specific activity)	Actual percent of arginine converted to urea in the cells during growth†
0. 1	0. 6	0. 01
1. 0	1. 4	0. 30
10. 0	2. 6	15. 10

HeLa-S_3 cells were grown for four generations in medium containing the specified arginine concentrations. The medium was then replaced with 100 ml of medium (2×10^5 cells/ml) containing guanido-C^{14}-L-arginine (Chemtrac Corporation, Cambridge, Mass.), specific activity 0.01 mc per mM at the specified concentrations. The cells were grown for an additional 24 hours, removed by centrifugation, an aliquot assayed for arginase as described in the text, and the protein of the medium was precipitated with a final trichloroacetic acid concentration of 10 percent. The protein-free supernatant fluid was extracted with ether to remove the trichloroacetic acid. The *p*H was adjusted to 6.6 with 1 M potassium phosphate, *p*H 6.6. A 1 ml aliquot was incubated with 5 mg urease (Sigma Chemical Co., Type V urease) for 60 minutes. The $C^{14}O_2$ liberated by the action of urease and collected and counted, as described previously (*7*), indicated the amount of urea formed from arginine during the growth of the cells.

*Extracts of HeLa-S_3 cells measured under optimal conditions of *p*H, manganese and L-arginine concentrations, expressed in terms of enzyme specific activity, *i.e.*, μmoles urea formed per hour per mg protein.

†As measured by formation of urea during growth.

in cultured cells indicative of repression phenomena have also been demonstrated for glutamyl transferase by DeMars (*21*) and for aspartate transcarbamylase by Ennis and Lubin (*22*). These studies indicate that the potential for repression phenomena exists under certain conditions in mammalian tissues. In the present study the repression phenomenon could be demonstrated only when growth was limited by the specific repressor, *i.e.*, arginine, which indicates that under normal growth conditions argininosuccinate synthetase and argininosuccinase are fully repressed. Ennis and Lubin (*22*) have come to the same conclusion in their studies with aspartate transcarbamylase. Other examples of repression in mammalian cells may well exist, and will probably be demonstrated only under conditions in which the biosynthetic product can be made to limit growth.

The studies with arginase demonstrated two separate and additive factors that resulted in the accumulation of increased enzyme activity: an effect of the substrate, arginine, and of a metal required for enzyme activity, manganese. The increased enzyme activity resulted from an increased content of enzyme protein caused by *de novo* protein synthesis. The results indicate that so-called enzyme induction in mammalian systems does not necessarily signify an increased rate of enzyme synthesis. In view of the general phenomenon of protein turnover in animal tissues and cell cultures, the possibility that the induction results from stabilization of the enzyme protein must always be considered. Changes in enzyme levels in mammalian tissues by a mechanism involving stabiliza-

tion of proteins, *i.e.*, prevention of degradation, may represent a common and significant mechanism for mammalian adaptation in general. Such a stabilization phenomenon has been shown to exist with rat liver arginase (*14*) and has been suggested as the mechanism for the increased activity of thymidylate kinase as produced by thymidine administration (*23*).

These studies also indicate the potential significance of small amounts of metal ions, in establishing the enzymatic properties of cell cultures, which may be introduced into the culture medium in ways unsuspected by the investigator. Thus Klein (*4, 24*) demonstrated a stimulation of arginase activity in several cell lines by yeast ribonucleic acid (RNA). In view of the effects of manganese demonstrated in these studies, and the known contamination of RNA preparations by divalent metals, including manganese (*25*), the RNA effect may well be the result of the contaminating metal present.

SUMMARY

Arginine in cell cultures can be synthesized from citrulline by a two-enzyme pathway, involving argininosuccinate synthetase and argininosuccinase, and can be degraded by arginase, thereby allowing for an evaluation of the existence of repression (biosynthetic pathway) and induction (degradative pathway) as metabolic control mechanisms in mammalian cell cultures.

A phenomenon analogous to classical bacterial repression was demonstrated in several continuous cultures with the arginine biosynthetic pathway. When HeLa, KB, or L-929 cells were grown in concentrations of citrulline or arginine that produced limitation of growth, the specific activities of argininosuccinate synthetase and argininosuccinase were 2-fold to 15-fold greater than when the cells were grown in excess arginine. The extent of change in enzyme levels was similar for both enzymes, *i.e.*, they were coordinated.

The level of arginase activity in HeLa-S_3 cells can be augmented by two separate, additive factors: 1) increase of arginine concentrations in the medium and 2) addition of manganese to the medium. An analysis of the time course of increases in arginase produced by arginine and manganese indicates that the effects are different. With the use of an antibody that specifically precipitates arginase of HeLa-S_3 extracts, both effects have been found to result from *de novo* protein synthesis. Arginase in cell cultures had a more rapid turnover, *i.e.*, degradation, than total cell protein. The addition of manganese to the medium stabilized the arginase molecule, which produced an accumulation of newly synthesized arginase by decreasing enzyme breakdown.

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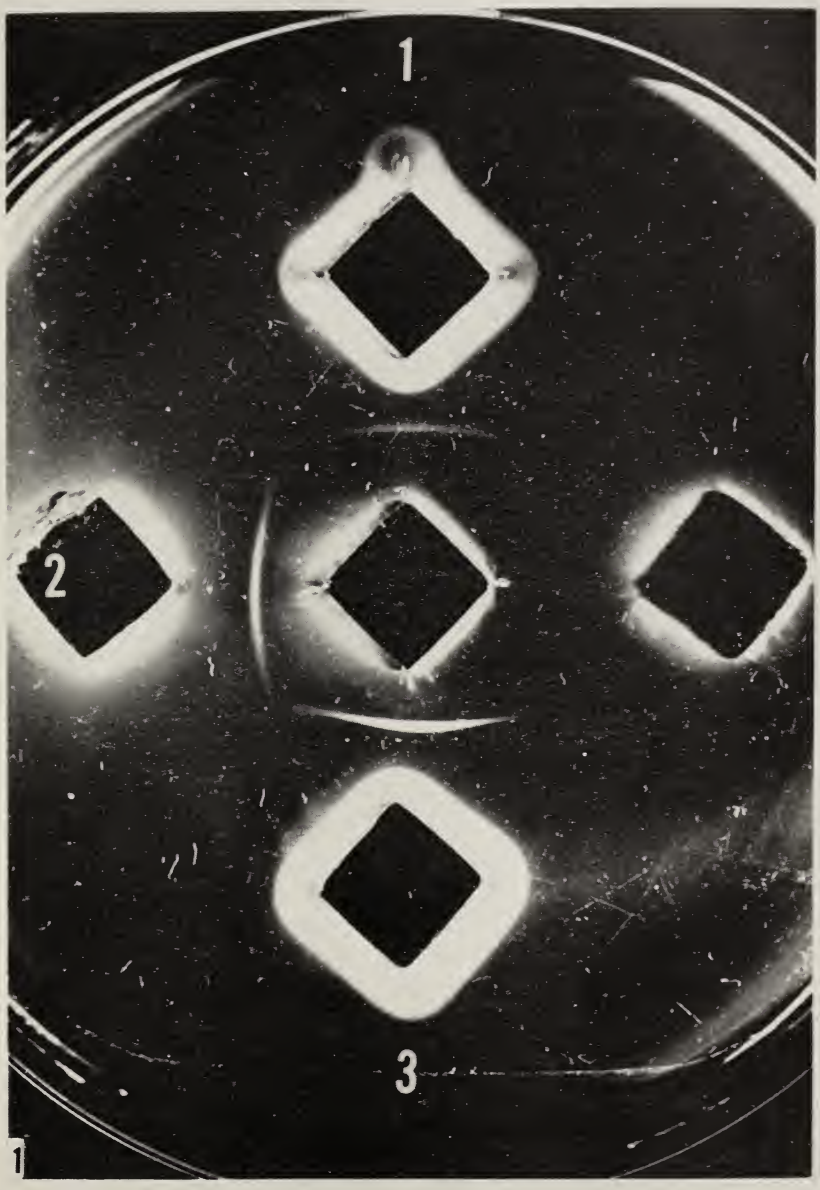
PLATE

Plate 19

Figure 1.—Ouchterlony diffusion pattern of antiarginase with rat liver arginase and HeLa-S_3 extracts. HeLa-S_3 extracts were prepared from cells grown in 0.1 mM L-arginine (*well 1*), or 2.0 mM L-arginine and 0.1 mM manganese chloride (*well 3*). *Well 2* contains highly purified rat liver arginase (*14*) similar to that used to immunize the rabbit. *Center well* contains the rabbit antiarginase antiserum. Photograph was made 21 days after introduction of the samples.

HELA-S_3 Extract: Specific Arginase Activity:
47μ Moles urea/Hr/Mg Protein



HELA-S_3 EXTRACT: SPECIFIC ARGINASE ACTIVITY: 550μ MOLES UREA / MG. PROTEIN

REGULATION OF ENZYME SYNTHESIS IN CULTURED CELLS[1,2]

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The regulation of protein synthesis in animal cells is of special interest since the many cell types in a single organism may possess an identical genetic constitution but exhibit different phenotypic characteristics. We studied cultured cells rather than intact animals or tissues to minimize the complexities of the experimental system. It was considered essential to have a system permitting repetition of the bacterial work (*1*), and cell strains offered the most promising approach.

Numerous reports of enzyme induction in cultured cells have appeared in the literature, beginning with the report of Burkhalter, Jones, and Featherstone, in 1957 (*2*). These experiments were done with freshly explanted material, and most of the early attempts to demonstrate similar systems in cell strains were unsuccessful. We were unable to observe any inductive or repressive effect in the first 10 enzyme systems investigated. Now, however, several useful systems have been found. It seems that high concentrations of inducers must be used and that induction may follow a lag of 1 to 7 days. The first system amenable to study was discovered by DeMars (*3*) who showed that D-glutamyl transferase increased in HeLa cells when they were grown in a medium containing a high concentration of glutamic acid instead of glutamine. We have studied a similar system in strain L cells (*4*). It is readily manipulated and we have therefore used it in attempts to answer some questions relating to the regulation of protein synthesis. Some of our results are referred to later.

An inducible rather than a repressible system seemed desirable for some purposes. We believed that such a system could be found in animal cells, as a few years ago we observed that in strain L cells some of the enzymes involved in deoxyribonucleic acid (DNA) synthesis, the thymidine kinases, increased prior to DNA synthesis and diminished afterwards (*5*). We showed that the level of the enzymes could be maintained if thymidine was added to the medium. Assay of these enzymes is tedious and therefore we have not investigated this system further. Cox

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and MacLeod (*6*) discovered that alkaline phosphatase increased in certain HeLa cells when prednisolone was added to the medium, a finding we have verified. Later, Cox and Pontecorvo (*7*) reported that an increase of the same enzyme could be obtained in skin fibroblasts when organic phosphates were added to the medium.

Most of these systems possessed unsatisfactory properties for our studies. We therefore examined many other enzymes in 6 different cell strains, taking samples for assay at intervals over a period of months in the hope that variations in the cellular content of certain enzymes might give us a clue to adaptive behavior (*8*). The specific activity of many of the enzymes fluctuated over a range of fivefold to tenfold (table 1). We investigated these to determine whether some of them could be induced or repressed and were successful in disclosing several systems (table 2). β-Glucuronidase could be repressed by potassium glucuronate in HeLa cells grown in the absence of glucose. We had previously observed (*9*) that, if cells were maintained under low oxygen tension for some time, respiration remained depressed for some hours before eventually returning to normal. Because of this, we investigated some enzymes of the Krebs cycle and found that malic dehydrogenase was greatly reduced in cells that had been exposed to partial anaerobiosis. Other less specific causes of fluctuation were also found. After trypsinization there is about one tenth the amount of acid phosphatase in strain L cells as before trypsinization, and the enzyme reaccumulates in the course of the next few days.

TABLE 1.—Range of activities of certain enzymes in cell strains

Enzyme	Specific activity (μM substrate attacked/min/100 mg protein nitrogen)					
	HeLa	HLM	Strain L	Y5	L5178 (cultured)	L5178 (ascites)
Alkaline phosphatase	10–110	120–260	0	0	0	—
Glucose-6-phosphatase	0. 25–2. 0	2–6	Trace	Trace	Trace	—
β-Glucuronidase	0. 25–1. 6	0. 75–0. 95	0. 4	0. 6–0. 8	0. 4–1. 8	—
Arginase	0–1. 6	0	0	0	Trace	1. 5–11
D-Glutamyl transferase	1. 8–3. 3	0. 8–3. 2	0. 5–1	0. 5–7	1. 5–2. 5	

The most potentially useful inducible system involved enzymes of the urea cycle. Some time ago Klein (*10*) reported that arginase increased in cultured cells when arginine and yeast ribonucleic acid (RNA) were added to the medium. In reviewing the literature we found variations in the amount of arginase in cultured cells (*11*). Furthermore, we found that L5178 cells exhibited little arginase activity when grown in culture but possessed high enzyme activity when grown as an ascites tumor. Consequently, we studied enzymes of the urea cycle and obtained results (*9*) similar to those recently reported by Schimke (*12*)

TABLE 2.—Conditions specifically influencing the activities of certain enzymes in cell strains

Enzyme	Cell	Conditions	Enzyme activity (units/10^6 cells)
β-Glucuronidase	HeLa	Eagle's medium (C)	0.92
		C—glucose + 2 mM K glucuronate	0.19
Malic dehydrogenase	HLM	Incubated in air	113.00
		Incubated in nitrogen, 60 hours	2.00
Acid phosphatase	L	1 Day after trypsinization	1.00
		6 Days after trypsinization	11.00
Arginase	HeLa	Eagle's medium (C), 8 days	0.0021
		C + 10^{-2} M arginine + 10^{-2} M citrulline, 8 days	0.0386
Glutamyl transferase	L	Eagle's medium (C), 48 hours	0.020
		C—glutamine + 20 mM glutamate, 48 hours	1.187
Alkaline phosphatase	HeLa	Eagle's medium (C), 10 days	0.54
	P	C + prednisolone (1 μg/ml), 10 days	4.80

CONDITIONS FOR ARGINASE INDUCTION

The conditions for induction of arginase in HeLa cells seem to be basically simple. If cells are grown in a high concentration of arginine for several days, the amount of arginase increases 5-fold to 20-fold. Better induction was obtained if citrulline, as well as arginine, was added to the medium (table 3). We believe there is a limit to the rate of entry of arginine into the cell and that the addition of citrulline probably helps to raise the concentration of the amino acid in the pool. We have been unable to obtain direct evidence for this, but there is indirect evidence from the behavior of L5178 cells. These cells have a very low arginase content when cultured *in vitro* but when grown as an ascites tumor in mice the intracellular arginase content is very high. By inoculating mice with large numbers of cultured L5178 cells, we showed that the amount of enzyme increases 10-fold to 20-fold in 24 hours (table 4) and up to 100-fold in 48 to 72 hours. When the cells are removed from the animal and again cultured, the enzyme is diluted out progressively and eventually after a few divisional cycles returns to the low level characteristic of cells in culture (table 5). Under these conditions the increased synthesis of the enzyme is much more dramatic than in the HeLa system. In cells grown as an ascites tumor the intracellular arginine content is high (57×10^{-17} mole/cell), whereas it is quite low in those grown *in vitro* (5.5×10^{-17} mole/cell). Apparently, in this system one of the main requirements for enzyme induction is an adequate concentration of the substrate inside the cell. Therefore, we tried to induce arginase in L5178 cells by adding arginine to the medium, but we have

obtained only small increases in arginase, though our experiments included the substitution of tumor fluid for ordinary culture medium. Because of these result with L5178 cells, we then tried to treat HeLa cells in various ways to facilitate the entry of arginine into the cells, but with little success. We found that when cells were grown in low oxygen tension the amount of arginase fell, though it could still be induced by increasing the concentration of arginine in the medium (table 6). This perhaps suggests that an energy-requiring pump mechanism is involved in the entry of arginine into the cell and that this can be prevented by inhibiting the respiratory pathways.

TABLE 3.—Induction of arginase in HeLa cells

Addition	Arginase activity (units/10^6 cells)
None	0. 0021
Arginine 10^{-2} M	0. 0070
Citrulline 10^{-2} M	0. 0063
Arginine 10^{-2} M + citrulline 10^{-2} M	0. 0386

TABLE 4.—Increase in arginase activity of cultured L5178 cells after inoculation into mice

Time after inoculation (hr)	Arginase activity (units/μg DNAP)
0	0. 0059
12	0. 02
24	0. 058

TABLE 5.—Disappearance of arginase from L5178 ascites cells after cultivation *in vitro*

Time after inoculation (hr)	Total deoxyribonucleic acid phosphorus per sample (μg)	A	B	A × B	Relative amount of total arginase
		Relative amount deoxyribonucleic acid	Enzyme units per μg deoxyribonucleic acid phosphorus		
0	76	1. 00	0. 016	0. 16	1. 0
18	134	1. 76	0. 0078	0. 014	0. 9
48	703	9. 20	0. 0023	0. 021	1. 3

TABLE 6.—Induction of arginase in HeLa cells grown for 4 days in anaerobic conditions, medium supplemented with ribose

Gas phase	Addition	Enzyme activity (units/million cells)
Air/CO_2	—	0. 00124
Nitrogen/CO_2	—	0. 00026
Nitrogen/CO_2	Arginine 10^{-2} M Citrulline 10^{-2} M	0. 00555

CONDITIONS FOR REPRESSION OF D-GLUTAMYL TRANSFERASE

The glutamyl transferase system is a repressible rather than an inducible system, since the level of this enzyme is controlled by its end product, glutamine. The repression is reproducible, and the changes in the enzyme levels occur fairly quickly. Consequently, it offers a suitable system for investigation of the regulatory mechanism.

In strain L cells glutamyl transferase exhibits another useful property: It disappears actively and rapidly when glutamine is restored to the medium (*13*). In the HeLa system originally described by DeMars (*3*) the enzyme disappeared by dilution. We have confirmed DeMars finding and it is clear that there is a real difference between the two types of cells and probably between the two species from which the cells are derived. Our evidence suggests that the enzyme is inactivated by oxidation rather than by proteolytic activity (*13*). Because of the active disappearance of the enzyme, it is possible to obtain a stable base line rather easily and to recognize endpoints quickly.

ROLE OF RIBONUCLEIC ACID IN REGULATION OF PROTEIN SYNTHESIS

There is good evidence that messenger RNA has a very important role in the regulation of the rate of protein synthesis in bacterial cells (*14*). In animal cells the evidence is much less definite, but it is reasonable to assume that the two systems have common characteristics. Certain discrepancies between the bacterial and animal cell systems should, however, be noted at the outset. 1) It is unusual for the enzyme to be completely absent from uninduced animal cells if sufficiently sensitive methods are used. 2) The change in activity from the uninduced to the induced state is rarely more than tenfold and only exceptionally as high as 100-fold. 3) The time course of induction is much slower in animal cells and may often require days instead of hours or minutes. It may require an exposure of 6 to 10 days to induce a striking increase in alkaline phosphatase, acid phosphatase, or arginase in strain L and HeLa cells (table 2). Even the rapidly synthesized enzymes, glutamyl transferase and arginase (in L5178), continue to accumulate for 48 to 72 hours. It is not yet possible to obtain sufficiently precise information about the minimum lag period before extra enzyme synthesis commences, but judging from our available data it is probably about 2 to 4 hours.

The first question is whether active protein and RNA synthesis are required for the increased enzyme activity. Since the amounts of new protein formed in all of these systems are relatively small, at present it is possible only to investigate this question with specific metabolic inhibition. Unfortunately, irreversible damage results when cells are exposed to the inhibitors for some hours, and it is nearly always essential to try to conclude the experiments within 24 to 48 hours. This has limited our

TABLE 7.—Effect of metabolic inhibitors on derepression of glutamyl transferase in strain L cells

Addition	Incubation time (hr)	Glutamyl transferase (units/10^6 cells)	Esterase (units/10^6 cells)
0	0	0. 013	0. 019
0	48	0. 121	0. 021
2×10^{-4} M 8-Azaguanine	48	0. 013	0. 009
2×10^{-4} M *p*-Fluorophenylalanine	48	0. 017	—
2×10^{-6} M 5′-Fluorodeoxyuridine	48	0. 086	—

investigations to derepression of glutamyl transferase. We have found (*13*) that inhibition of DNA synthesis by 5-fluoro-2′-deoxyuridine (FUDR) does not prevent an increase of enzyme activity when glutamine is omitted, but, if azaguanine or fluorophenylalanine is present in the medium, the increase does not occur. Esterase activity measured simultaneously is not profoundly affected, and therefore we conclude that the extra glutamyl transferase depends on *de novo* RNA and protein synthesis (table 7).

We have attempted to obtain more information on the role of RNA in the control of protein synthesis by adding RNA extracted from induced cells to a protein-synthesizing system of the Nirenberg-Ochoa type (*15*, *16*). High levels of amino acid incorporation can be promoted in these systems by addition of RNA or synthetic polynucleotides (*see* table 11), but despite intensive investigation we have obtained equivocal results on the promotion of the synthesis of catalytically active enzymes. Apparently, one difficulty is to keep the long molecules (of messenger RNA?) intact.

BEHAVIOR OF NUCLEAR RIBONUCLEIC ACID IN ANIMAL CELLS

We have indicated that after inhibition of synthetic processes in strain L cells by FUDR, there was no simple kinetic relationship between nuclear RNA and cytoplasmic RNA (*17*). Our recent results (*18*) agree with those of Harris (*19*) who found no good evidence that rapidly labeled nuclear RNA becomes associated with ribosomes. These observations are at variance with the findings of other workers (*20*) who have reported an accumulation of 28S and 16S RNA, associated with disappearance of rapidly labeled 44S RNA in HeLa cells. We believe that this discrepancy can be explained by the fact that actinomycin D, at the low concentrations used by these workers to inhibit DNA-primed RNA synthesis, takes some time to achieve full inhibition of RNA synthesis. During this time a stable slowly labeled RNA accumulates while the unstable rapidly labeled RNA decays.

With strain LS cells, a variant of strain L which grows spontaneously in suspension, some hours are required to accomplish complete inhibition

TABLE 8.—Inhibition of nuclear ribonucleic acid synthesis in strain LS cells by 2 μg per ml actinomycin D

Time after addition (hr)	Relative amount of uridine incorporation in 1 hour
0	100
1	46
2	22
3	13
4	10
5	8
6	5

of nuclear RNA synthesis in the presence of 2 μg per ml actinomycin D (table 8), whereas, at a concentration of 100 μg per ml, nuclear RNA synthesis is completely inhibited in a few minutes (table 9). The inhibition is not reversible and hence it is possible to employ a pulse of the inhibitor. Using this system we found that the disappearance of activity from rapidly labeled nuclear RNA was not accompanied by an accumulation of activity in microsomes. Harris has reported a similar finding (*21*). We also found that some slowly labeled RNA continued to accumulate in both the nucleus and the cytoplasm of cells inhibited with actinomycin D. The actinomycin D-resistant RNA labeling occurred in the nuclear and cytoplasmic microsomes (table 10). Since no commensurate accumulation of activity occurred in the supernatant fractions, it is unlikely that this is due to terminal addition of cytosine in sRNA.

It seems reasonable that the rapidly labeled RNA of the nucleus is a type of messenger RNA, especially since it occurs in the RNA-DNA-protein complex that can be extracted from nuclei with molar sodium chloride (*22*). We are investigating the possibility that a primary messenger may be copied by an RNA-primed RNA-synthesizing system to form a secondary messenger, actually involved in protein synthesis. This concept is attractive since it provides an explanation for the ready multiplication of RNA viruses in animal and plant cells. It also provides an amplification system for protein synthesis and permits controlling mechanisms to operate at another level.

To obtain more direct evidence concerning the location of the messenger in animal cells, we have tried using different RNA fractions to prime amino acid incorporation in a subcellular system. However, as shown in table 11, and as supported by the work of others, there is little difference in the priming capacity of different types of RNA.

TABLE 9.—Inhibition of nuclear ribonucleic acid synthesis in strain LS cells by 100 μg per ml actinomycin D

Time after addition (min)	Relative amount of uridine incorporated in 10 minutes
0	100
5	8
20	1

TABLE 10.—Effect of a pulse of 100 μg per ml actinomycin D on incorporation of H^3-uridine into different ribonucleic acid fractions in strain LS cells

RNA fraction	Total activity in fractions								
	H^3-uridine only (hr)			Actinomycin D added 30 minutes after H^3-uridine (hr)			Actinomycin D added before H^3-uridine (hr)		
	0	1	2	0	1	2	0	1	2
Nucleolar I	60	100	85	41	37	32	8	6	8
Nucleolar II	144	182	176	110	66	58	16	13	16
DNA/RNA	46	37	38	29	36	19	4	4	6
Nuclear sap	54	75	75	12	18	21	7	5	9
Nuclear ribosomes	14	19	33	7.3	9.6	11.8	5	6.5	10.2
Cytoplasmic ribosomes	295	395	600	235	196	200	136	150	230

CONCLUSIONS CONCERNING CONTROL OF PROTEIN SYNTHESIS IN ANIMAL CELLS

The first question concerning protein synthesis in animal cells is whether it is basically the same or different from the more thoroughly investigated mechanisms in bacterial cells.

In enzyme induction there are apparently clear-cut differences in both the rate and degree of inducibility. These may well reflect differences in basic mechanisms, yet may merely reflect the difficulty of manipulating the internal environment of the animal cell, since the systems so far studied are not good examples of "enzymes of gratuity." Thus these differences should be interpreted cautiously until more information is available.

There is an apparent discrepancy between the accepted pattern of behavior of RNA in bacterial cells and that in animal cells. It may be, as Harris suggests (*19*), that the interpretation of the bacterial findings is wrong, but it is perhaps more likely that in more highly evolved cells additional refinements have been added to the basic mechanisms present in all cells; these await elucidation.

A question which inevitably arises is: What is the connection between these phenomena and cytodifferentiation? Clearly all cytodifferentiation might be explained in terms of interlocking feedback reactions (*23*) involving simple repressions and inductions similar to those discussed.

TABLE 11.—Stimulation of C^{14}-phenylalanine incorporation into acid-soluble material by nucleic acid fractions

Type of ribonucleic acid	Concentration (μg/ml)	Counts per mg of protein	
		I	II
Sucrose gradient fractions: 4S	15	1,020	800
Sucrose gradient fractions: 28 + 16S	34	850	620
Sucrose gradient fractions: 44S	15	400	530
Nucleolar I	50	490	710
Nucleolar II	50	580	620

However, it is important that certain differences among cells can persist in long-term cultures, even in proteins whose production can be specifically regulated by manipulation of the environment (table 1). Enzyme induction and repression undoubtedly provide tools for the study of the regulatory mechanisms in differentiation, but it would seem to be premature to draw more far-reaching conclusions.

SUMMARY

Several inducible or repressible enzymes were found in cultured animal cell strains. In some the stimulus was relatively nonspecific, *e.g.*, induction of acid phosphatase in strain L cells after trypsinization and repression of malic dehydrogenase in anaerobic conditions. In a few, however, the stimulus was either the substrate or the end product of the enzyme reaction. Two systems, the induction of arginase by arginine and the repression of D-glutamyl transferase by glutamine, are suitable for the study of the regulatory mechanisms and were used mainly in this work. Arginine can be induced in HeLa cells by increasing the concentration of arginine and/or citrulline in the medium and in L5178 cells by transplanting them to mice. Induction of arginase was limited by the entry of arginine into the cell, and cells growing as ascites tumors were apparently able to concentrate the amino acid more effectively than cultured cells. D-Glutamyl transferase was repressed by glutamine in the medium. In nearly all the systems investigated, the enzyme was measurable in both the induced and uninduced state and the change in activity was rarely more than tenfold. There was usually a lag period varying from hours to days before a change in activity was detectable and it often was not completed until the cells had been exposed to the new conditions for several days. After removal of glutamine, reappearance of glutamyl transferase was prevented by azaguanine and fluorophenylalanine but not fluorodeoxyuridine. RNA and protein synthesis are thus implicated in the increase in enzyme activity. Therefore, the behavior of RNA in cultured cells was investigated. The rapidly labeled nuclear RNA apparently does not become associated with cytoplasmic microsomes, though a more slowly labeled RNA does. From the kinetic data and the preliminary information about nuclear RNA, it is considered that the regulation of protein synthesis in animal cells may differ in some respects from similar phenomena in microorganisms.

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HEPATOMAS IN TISSUE CULTURE COMPARED WITH ADAPTING LIVER *IN VIVO* [1, 2]

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It has long been apparent to the biochemist that in the mammal the hepatic cell serves a unique function in governing the relationship of the external environment to the internal "milieu." As a necessary consequence of this relation, the enzymatic apparatus of the hepatic cell must possess considerable flexibility to cope successfully with the myriads of molecular species that gain entrance to the blood stream either directly by injection or indirectly through the gastrointestinal tract. Demonstration of this metabolic flexibility of the hepatic cell was hinted at by early workers who studied the effect of dietary intake and compostion on the enzymatic content of the liver (*1*). But the degree and specificity of this flexibility was not appreciated by biochemists until Knox (*2*) reported that tryptophan administration *in vivo*, either parenterally or orally, resulted in a rapid rise in the level of hepatic tryptophan-pyrrolase activity. This finding, widely confirmed and investigated (*3*, *4*), suggested that alterations in liver enzymes may be specifically controlled by small molecules by a process like that of enzyme induction in microorganisms (*5*). Later studies by Civen and Knox (*6*) showed the structural specificity of the inducing amino acid, and Feigelson and Greengard (*7*), as well as Nemeth and de la Haba (*8*), demonstrated that the induction of tryptophan pyrrolase probably results from a net synthesis of the enzyme, which thus relates the process more closely to the microbial system. However, one distinct difference between the mammalian and bacterial sytems is that corticosteroids will also induce the enzyme in liver. This induction is a result of net enzyme synthesis (*7*), but is not accompanied by altered tryptophan levels in liver (*9*) and appears to act by a different mechanism from the substrate induction. Furthermore, recent studies by Greengard and Acs (*10*) showed actinomycin D inhibits the cortisone induction in the adrenalectomized rat but not the tryptophan induction of tryptophan

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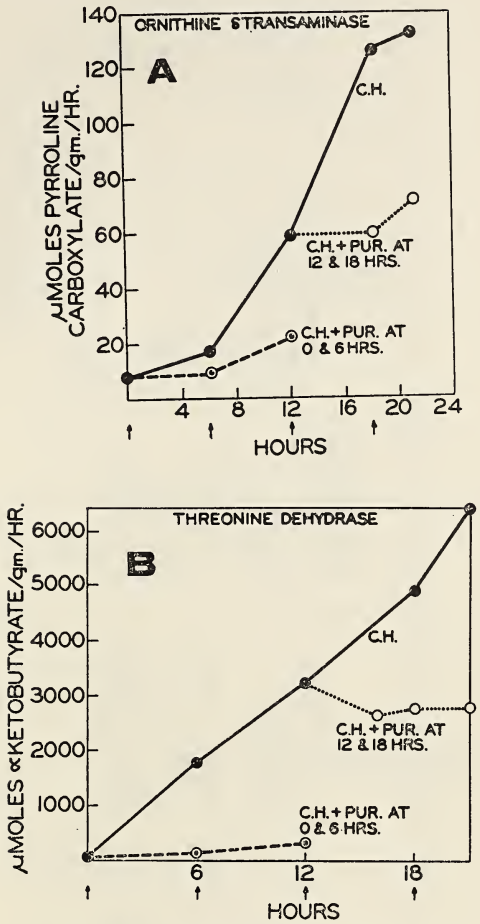
pyrrolase, which suggests that the steroidal but not the amino acid stimulation of enzyme synthesis depends on concomitant ribonucleic acid (RNA) synthesis. Other enzymes, notably tyrosine α-ketoglutarate and glutamic-pyruvic transaminase, are also induced by cortisone administration (*11*, *12*). This induction results in net enzyme synthesis (*12*, *13*) and is inhibited by actinomycin D (*10*). However, the induction of tyrosine transaminase is purely hormonal, since the substrate tyrosine induces the enzyme in the intact but not in the adrenalectomized animal (*11*).

Dietary protein or carbohydrate is also capable of inducing specific enzymes, as alluded to earlier. In particular, Tepperman and Tepperman (*14*) observed a marked increase in glucose-6-phosphate dehydrogenase in liver after fasting rats for several days and then feeding them a 60 percent glucose diet. Moreover, the 10- to 20-fold increase in glucose-6-phosphate dehydrogenase activity, which occurs in control animals, is inhibited in animals receiving X radiation or puromycin (*15*). Dietary protein is also capable of inducing the enzyme threonine dehydrase (*16*). In recent studies in this laboratory, we induced threonine dehydrase, an enzyme of the cell sap, and ornithine transaminase, an enzyme of the mitochondrial sap (*17*), by the forced feeding of hydrolyzed casein to protein-depleted rats (*18*). These results are seen in text-figure 1. Ornithine transaminase is induced 10- to 20-fold in 24 hours under these conditions, whereas threonine dehydrase increases more than 100-fold in the same time period. The induction is halted or prevented by the administration of puromycin (text-fig. 1), which thus indicates that the process reflects net enzyme synthesis. As yet, we have been unsuccessful in inducing either enzyme by its substrate alone.

Several examples of control mechanisms in liver analogous to product repression in bacteria have been discovered. Walker reported that dietary creatine caused a marked fall in the level of liver arginine-glycine transamidinase in the chick (*19*). The same occurs in the kidney of the mammal (*20*), which has no hepatic transamidinase. The removal of creatine from the diet of the chick results in a restoration of transamidinase activity that is inhibited by ethionine (*21*), which thus indicates a function of enzyme synthesis in the derepression phenomenon.

In mammals, a phenomenon of apparent product repression is the inhibition of cholesterol biosynthesis by dietary cholesterol. The feeding of a diet containing 2 percent cholesterol to rats for 2 days results in a 95 to 98 percent reduction in the incorporation of acetate-C^{14} into cholesterol in slices of liver incubated *in vitro*. Recent studies have shown that this is probably a result of a decrease in an enzyme involved in the production of mevalonic acid from β-hydroxy-β methyl glutaric acid (*24*).

Another type of repression of protein synthesis extensively studied in microorganisms, but not yet reported to occur in mammals, is glucose repression of catabolic enzymes. Recently we showed that this phenomenon occurred in rat liver (*18*) when the two enzymes ornithine transaminase and threonine dehydrase were induced (text-fig. 2). Concomitant



TEXT-FIGURE 1, *A* AND *B*.—Effect of puromycin injection on the rapid induction of ornithine-δ-transaminase and threonine dehydrase. C.H. = casein hydrolysate; Pur. = puromycin.

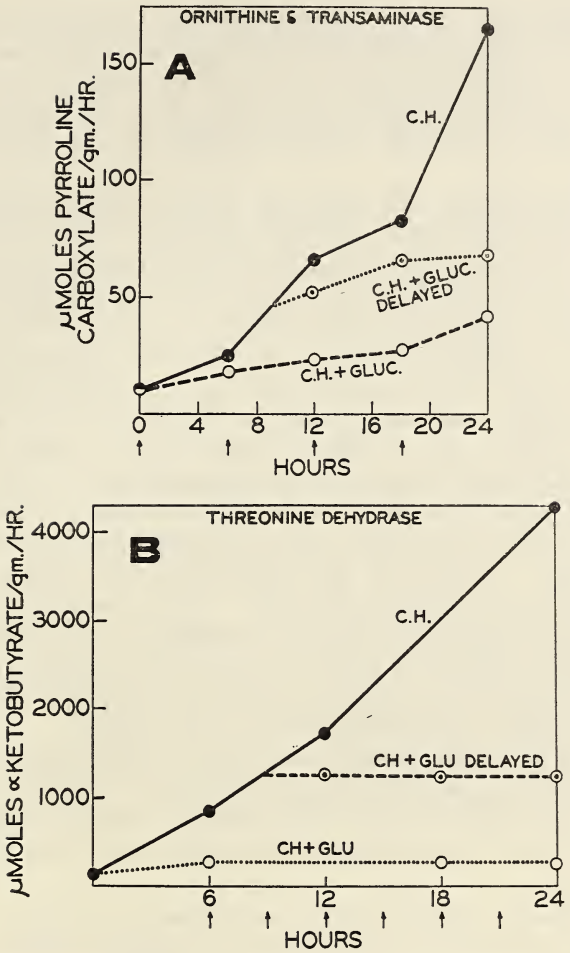
Casein hydrolysate was given to the rats by stomach tube at the times designated by *arrows* along the abscissas. Zero time values are from rats killed just before the first feeding.

administration of glucose virtually abolishes the dietary induction of these two enzymes. If the glucose administration is delayed until 9 hours after the induction is begun, an abrupt cessation of enzyme synthesis occurs. The effect is not caloric nor is it due to a blockage of absorption or an unavailability of adenosine triphosphate (*18*).

Thus in mammalian liver there are a number of examples of the environmental control of enzyme levels, and these probably reflect enzyme synthesis when the increase is blocked by puromycin. Those discussed here are outlined in table 1. There are other known and undoubtedly many unknown examples of the control of enzyme synthesis in liver that

TABLE 1.—Systems controlling enzyme synthesis in rat liver *in vivo*

Type	Example	Stimulus	Time for maximal induction or repression	Associated process	Reference
Substrate induction	Tryptophan pyrrolase	Tryptophan	4 Hours	Protein synthesis	(*7, 8, 10*)
Hormonal induction	Tyrosine transaminase	Cortisone	3–4 Hours	Protein and RNA synthesis	(*10, 13*)
Dietary induction	Glucose-6-phosphate dehydrogenase	Dietary carbohydrate	18–48 Hours	Protein and RNA synthesis	(*14, 15*)
	Threonine dehydrase + ornithine transaminase	Dietary protein	6–24 Hours	Protein ± RNA synthesis	(*18*)
Product repression	Cholesterol biosynthesis	Dietary cholesterol	24–48 Hours	?	(*24*)
Glucose repression	Threonine dehydrase + ornithine transaminase	Dietary glucose	5–10 Minutes	Protein and RNA synthesis	(*18*)



TEXT-FIGURE 2, *A* AND *B*.—Effect of glucose feeding on the rapid induction of ornithine-δ-transaminase and threonine dehydrase. Gluc. = glucose. All rats were given casein hydrolysate, as in text-figure 1 *A* and *B*. Rats receiving glucose from zero time were given the sugar mixed with casein hydrolysate. Rats receiving glucose after a delay were given it separately at 9, 15, and 21 hours.

further allow the hepatic cell its required metabolic plasticity. Evidence has also indicated that biologic alteration of the liver *in vivo*, such as regeneration (*22*) and cirrhosis (*16*), will not alter its responses to several environmental stimuli. But what of the biologic alteration, neoplasia, in which one, if not *the* key defect resides in control mechanisms (*23*)?

CONTROL SYSTEMS IN HEPATOMAS *IN VIVO*

The systematic comparative study of enzymatic systems in hepatomas in relation to liver was first undertaken on a grand scale by the late Dr.

J. P. Greenstein (*25*). Since his pioneering efforts, numerous workers (*26*) have studied enzymatic differences between the normal and malignant hepatocyte. In particular, the liver-hepatoma system gave rise to the "deletion" hypothesis of carcinogenesis (*27*), which still provides probably the best working model for experimentation in the biochemistry of malignancy.

However, the biologic complications of this system, apparent to the pathologist for many years, were not obvious to the biochemist until recently. In the last decade it became evident that some hepatic carcinomas little resembled liver, whereas others closely resembled liver in their enzymatic capacities. For example, the Novikoff hepatoma had few liver-specific enzymes, whereas the Dunning hepatoma had several enzymes of liver that the Novikoff did not have, and the newly discovered "minimal deviation" hepatomas, such as the Morris 5123, had virtually all hepatic enzymes [table 2, (*28*)]. These 3 tumors differed markedly from each other in their morphology and biologic behavior (*29*); however, Hepatoma 5123 had almost all the biochemical characteristics of liver, including virtually no glycolysis (*30*), the h-proteins (*29*), and a normal karyotype (*29*). In addition, its ultrastructure was found to be very similar to that of the hepatic cell (*28*). Thus it was proved that the biologic criteria of malignancy could be satisfied with apparently minimal changes in the biochemistry of the converted cell. Probably only those biochemical changes closely associated with the malignant transformation itself were present in the minimal deviation hepatomas.

And what of the control of enzyme synthesis in these hepatomas? Early work on primary hepatomas indicated that no substrate induction of tryptophan pyrrolase occurred in the tumors, whether induction was carried out *in vivo* or in tissue slices *in vitro* (*16*). Later work with minimal

Table 2.—Enzymes of liver and hepatomas in the rat*

Enzyme	Liver	Morris 5123	Novikoff	Dunning
Glucose-6-phosphatase	+	+	−	±
Glycerophosphate dehydrogenase	+	+	−	+
Choline oxidase	+	+	−	+
Uricase	+	+	−	−
Thymine reductase	+	+	−	−
Uracil reductase	+	+	−	−
Thymidylic synthetase	+	+	+	+
Thymidine kinase	+	+	+	+
Glutamic dehydrogenase	+	+	−	+
Threonine dehydrase	+	+	−	−
Serine dehydrase	+	+	−	−
Tryptophan pyrrolase	+	+	−	±
Proline oxidase	+	+	−	−
Phenylalanine hydroxylase	+	+	−	
Arginase	+	+	−	
Ornithine transcarbamylase	+	+	−	
Fructose-1,6-diphosphatase	+	+	−	+
dCMP deaminase	−	−	+	−

*Plus sign denotes measurable activity; minus sign indicates that no activity can be obtained by the method of assay used (*29*).

deviation hepatomas gave essentially the same results with a few exceptions (*31*). The highly differentiated Reuber hepatoma H-35 exhibited a slight degree of substrate induction in the intact but not in the adrenalectomized host. Thus no hepatoma, primary or transplanted, has the capacity for pure substrate induction of this enzyme, a marked defect when compared to normal liver.

In contrast, tyrosine transaminase is at a very high level in these neoplasms growing in an intact host (*31*). Cortisone administration does not alter this level significantly, but adrenalectomy of the host causes a lowering of the level in the tumor to that found in the host liver. In this case, acute or chronic cortisone administration to the adrenalectomized animal resulted in a return to the high level seen in the tumor of the intact host (*31*). Only acute cortisone induces the enzyme in the liver, whether the host is adrenalectomized or not. Carbohydrate induction of glucose-6-phosphate dehydrogenase and protein induction of threonine dehydrase also are lacking in virtually all tumors studied thus far. In the Morris 7800, a high protein diet causes a considerable increase in threonine dehydrase of the tumor in intact but not in adrenalectomized animals, a situation much like that with tryptophan pyrrolase (*31*).

Recently Siperstein and his co-workers found that cholesterol feeding does not repress its synthesis by certain mouse hepatomas (*32*). In our laboratory, preliminary evidence suggests that glucose repression of threonine dehydrase induction may also be faulty in some hepatomas.[5]

Therefore, in every control system investigated thus far some defect appears evident in the hepatomas. The results to date are summarized in table 3. But what is the molecular basis for these defects? One approach is the use of cell-free systems (*33*); another is the use of tissue culture.

CONTROL SYSTEMS IN HEPATOMAS *IN VITRO*

Many authors have described the cultivation of cells from experimental hepatomas (*34*). However, relatively few have undertaken the rather crucial experiment of reinoculation into a suitable host to determine whether the cultivated cell is the same as the original explant. Early work in this laboratory, in conjunction with Dr. Earl Swim, showed it was possible to keep at least two rapidly growing, enzymatically and morphologically distinct hepatic carcinomas—the Dunning L-C18 and Novikoff hepatomas—in culture *in vitro* for extended periods without any appreciable alteration of their malignant potential or their enzymology or morphology *in vivo*. In later work, we explanted the Morris 5123 to glass and maintained the cultures for extended periods (*35*). Some of these were reimplanted in suitable hosts, and the tumors obtained were indistinguishable from the original tumor, insofar as the studies have been extended. These results are summarized in table 4. Investigation

[5] Peraino, C., and Pitot, H. C.: Unpublished observations.

TABLE 3.—Control systems in hepatomas *in vivo*

Type	Example	Stimulus	Hepatomas studied	Comparison with liver	Associated process	Reference
Substrate induction	Tryptophan pyrrolase	Tryptophan	All available	Absent or abnormal in hepatoma	Protein and RNA synthesis where occurs (?)	(*31*)
Hormonal induction	Tyrosine transaminase	Cortisone	All available	Increased "sensitivity" to hormone	Protein and RNA synthesis (?)	(*31*)
Dietary induction	Glucose-6-phosphate dehydrogenase	Dietary carbohydrate	5123	Absent	—	(*15*)
	Threonine dehydrase + ornithine transaminase	Dietary protein	5123, 7800, 7316, H-35, and 7794	Absent or abnormal in hepatoma	Protein and RNA synthesis	(*31, Footnote 5*)
Product repression	Cholesterol biosynthesis	Dietary cholesterol	Mouse hepatoma	Hepatoma synthesizes cholesterol regardless of dietary content	(?)	(*32*)
Glucose repression	Threonine dehydrase + ornithine transaminase	Dietary glucose	5123, 7793	Absent	—	(*Footnote*)

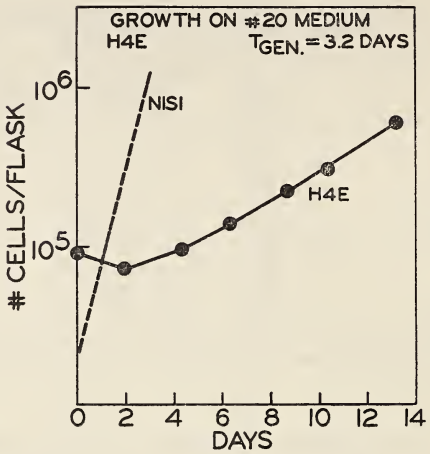
TABLE 4.—Enzymes of normal rat liver and the neoplasms produced by the cultured cell lines: H2, N1-S1, and D1-29*

Enzyme	Liver	H2 (Morris 5123)	N1-S1 (Novikoff)	D1-29 (Dunning)
Choline oxidase	+		−	+
Thymine reductase	+	+	−	−
Thymidine kinase	+	+	+	+
Threonine dehydrase	+	+	−	−
Glutamic dehydrogenase	+	+	−	+
dCMP deaminase	−	−	+	−
Proline oxidase	+	+		
Serine dehydrase	+	+		
Glucose-6-phosphatase	+	+		

*Plus sign denotes measurable activity; minus sign indicates that no activity can be obtained by the method of assay used.

of the karyotypes of the Morris 5123 and the 5123-H2 line from tissue culture indicated that the former possessed a normal diploid chromosomal complement, whereas the 5123-H2, which had been explanted to tissue culture and reimplanted in animals, had a mode of 43 or 44, *i.e.*, 1 or 2 greater than a normal diploid, 42. The 5123-H2 in culture was later lost due to overgrowth of a contaminating fibroblast population, which gave rise to sarcomas rather than carcinomas on reinoculation into animals. The original explants have been maintained *in vivo* by Dr. H. P. Morris, of the National Cancer Institute.

In August, 1961, Dr. P. A. Morse, of our laboratory, explanted a mince of the Reuber hepatoma H-35 to T-30 flasks, using a modified Eagle's medium containing 20 percent horse serum and 5 percent beef embryo serum (*35*). The cells, designated strain H-4, were carried with very slow growth through 5 subcultures and reinoculated into A × C strain rats in November, 1961. A typical H-35 tumor was produced and then explanted to T flasks (strain H4-II). After the second subculture, epithelial colonies began growing on the surface of the fibroblastic monolayer. Epithelial colonies were picked and carried through 3 "reclonings," to give strain H4-II-E-C3. All attempts at single-cell cloning failed. These epithelial colonies grew very slowly (generation time = 1 week) at high dilutions, but more rapidly in heavier populations or in the presence of fibroblasts in the mixed culture. After 3 serial colony clonings, the H4-II-E-C3 cell strain was free of fibroblast contamination and consistently gave typical Reuber H-35-type tumors on inoculation *in vivo*. This line, termed the H4-II-E, has remained stable for a year. Its generation time is about 3.2 days (text-fig. 3). Its appearance is that of rather typical epithelial colonies (fig. 1). Chromosomal studies showed the H4-II-E has a mode of 46, while the original H-35 hepatoma has a mode of 42–43. The morphology of the H-35 and of the H4-II-E, when reimplanted *in vivo*, is almost identical. The enzymology of the H-35 and of the H4-II-E as compared with liver is given in table 5. Several of the enzymes present in liver and the H-35 *in vivo* are absent from the H4-II-E *in vitro*, *i.e.*, tryptophan pyrrolase, glucose-6-phosphate dehy-



TEXT-FIGURE 3.—Growth curve of culture of H4-II-E. *Dotted line* shows the growth curve of the Novikoff hepatoma (N1-S1) for comparison.

drogenase, and proline oxidase. Also threonine dehydrase is at very low levels, within the limits of the assay. However, the hepatic "marker" enzymes, histidase, ornithine transaminase, tyrosine transaminase, thymine reductase, and glucokinase are present at measurable levels in the cultured cells. The absence of such an important enzyme as glucose-6-phosphate dehydrogenase is of interest because of its importance in the intermediary metabolism of carbohydrate. Previous studies in this laboratory (*15*) showed this enzyme was markedly affected by adrenal hormones.

With the existence in a virtually pure culture of a serially propagated cell line, which is a direct descendant of the Reuber hepatoma H-35, it became of considerable interest to determine whether systems controlling the levels of enzymes in liver or in hepatoma *in vivo* were operative *in*

TABLE 5.—Enzymes of liver *in vivo*, Reuber H-35 *in vivo*, and H4-II-E *in vitro**

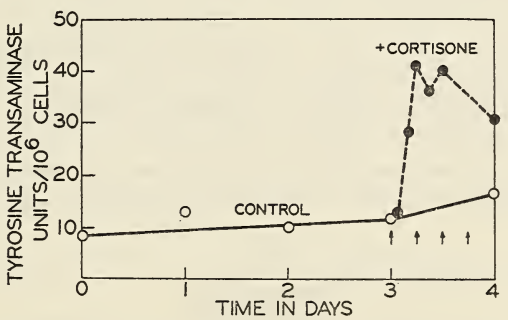
Enzyme	Liver	H-35	H4-II-E
Glucose-6-phosphate dehydrogenase	100–300	50–100	0
Tryptophan pyrrolase	2–4	0. 3–0. 6	0
Histidase	25–75	2–3	0–3
Threonine dehydrase	20–80	0–70	0–20
Proline oxidase	120–280	9–15	0
Ornithine transaminase	75–110	380–520	320–400
Tyrosine α-ketoglutarate transaminase	75–150	200–600	80–200
Hexokinase	15–30	10–30	18
Glucokinase	30–56	13–20	19
Thymine reductase	1. 6–2. 8	0. 14†	0. 01†

*All values are in μmoles product per g wet weight per hour. The values given are ranges and in all cases except the kinases of H4-II-E represent at least 3 values. Enzymes of the H-35 *in vivo* before and after tissue culture explantation are essentially the same.

†Approximate value.

vitro. Preliminary experiments were undertaken to demonstrate the induction of tryptophan pyrrolase *in vitro*. Cells were maintained for short periods in the presence of α-methyltryptophan [6] (10^{-4} M), an efficient inducer of tryptophan pyrrolase (*6*), with and without any cortisone (10^{-6} M) in the medium. No evidence of enzyme induction occurred. In addition, some cultures were maintained for periods up to 24 hours in the absence of glucose, but no change in the level of threonine dehydrase was elicited by this maneuver which was designed as an attempt to relieve the possible glucose repression of the enzyme. These findings *in vitro* correlated well with the data *in vivo*, which show that both tryptophan pyrrolase induction and glucose repression of threonine dehydrase are low in these hepatomas.

However, tyrosine transaminase was highly responsive to corticoids in the Reuber H-35 hepatoma and in all other hepatocellular carcinomas studied thus far (*31*). The data obtained both *in vivo* and *in vitro* are given in table 6. When the H-35 is maintained *in vivo* in intact hosts, the enzyme level is 5 to 20 times higher in the tumor than in the liver. Cortisone administration (25 mg/kg cortisone acetate, intraperitoneally) to the intact host induces the enzyme in the liver but has substantially no effect on the tumor. In the adrenalectomized host, however, the enzyme in the tumor is in the range of the host liver. Cortisone administration induces the enzyme in both liver and tumor. These findings are similar to those in other minimal deviation hepatomas (*31*). The last two lines of table 6 show the effect of cortisone administration *in vitro*. The control level is slightly higher than that in control host liver, but not so high as in the H-35 in intact hosts. If cortisone (10^{-6} M cortisone acetate) is added to the cultures 24 hours before the enzyme assay, a significant induction occurs *in vitro*. This induction may be followed in a time sequence as seen in text-figure 4. The enzyme is maximally induced at 6 hours after



TEXT-FIGURE 4.—Time curve of tyrosine transaminase levels per 10^6 cells in cultures of H4-II-E with and without cortisone. Modified Eagle's medium with 20 percent horse serum and 5 percent beef embryo serum. Each point represents the average of assays on 2 or 3 separate T-60 flasks. Cortisone acetate (10^{-6} M final concentration) was added at each *arrow*. *Vertical lines* represent the range of values.

[6] Kindly supplied by Dr. Karl Pfister, Merck Sharp & Dohme, Rahway, N.J.

TABLE 6.—Tyrosine transaminase in liver, H-35, and H4-II-E*

	Liver	H-35	H4-II-E
Control *in vivo*	15–50	200–600	
+ Cortisone *in vivo*	220–300	300	
ADRX *in vivo*	20–55	50–80	
+ Cortisone *in vivo*	120–450	130–300	
Control *in vitro*			80–200
+ Cortisone *in vitro*			400–680

**See* asterisk footnote table 5. All ranges represent at least 3 values.

the addition of cortisone to the medium. If cortisone is removed after 6 hours, the enzyme level remains high for at least another 18 hours even in the apparent absence of the hormone. Actinomycin D (0.5 μg/ml) added to the medium delayed the induction by 2 to 4 hours, but did not inhibit it at this level of drug.

Thus the Reuber H-35 hepatoma *in vitro* exhibits a corticosteroid induction of tyrosine transaminase. The kinetic characteristics of this induction seem to differ somewhat from those seen in liver *in vivo* in that after cortisone is metabolized and removed *in vivo*, the enzyme level rapidly returns to normal; *in vitro* with H4-II-E, the high level is maintained for at least 18 hours. This may be characteristic of the kinetics of tyrosine transaminase induction in this tumor, both *in vitro* and *in vivo*.

DISCUSSION

From the data available at present, hepatocellular carcinomas apparently possess a wide range of defects in the control of enzyme synthesis when compared with normal or host liver. A number, if not all, of these defects are also present in at least 1 hepatoma growing *in vitro*, which thus demonstrates that even when all the host-tumor interactions are removed, this basic abnormality of defective control is present.

As yet no adequate control for hepatomas *in vitro*, *i.e.*, liver *in vitro*, is available. However, with the advances in our knowledge of tissue culture, the day is not far off when the biochemical properties of normal and malignant liver growing *in vitro* can be rationally compared.

SUMMARY

With the microbial system as a model, several different methods of the environmental control of protein synthesis in mammalian liver *in vivo* have been discovered. Examples are substrate induction (tryptophan pyrrolase), product repression (cholesterol biosynthesis), hormonal induction (tyrosine transaminase), dietary induction (glucose-6-phosphate dehydrogenase, ornithine transaminase, threonine dehydrase), and glucose

repression (threonine dehydrase and ornithine transaminase). When these control mechanisms are studied in hepatocellular carcinomas growing *in vivo*, significant qualitative and quantitative differences are found in almost every case when compared with the same mechanism in liver.

When hepatocellular carcinomas are explanted to tissue culture, they retain their malignant capacity, and when reimplanted *in vivo*, result in neoplasms that appear to be almost indistinguishable biochemically and morphologically from the original explant. However, cultivation *in vitro* does alter the karyotype slightly in most cases. In addition, the neoplasms obtained on reinoculation of the cultured cells usually display a more rapid growth rate *in vivo* when compared to that of the original explant. Enzyme induction by corticosteroids is retained in at least one hepatocellular carcinoma *in vitro*, but, as yet, no substrate induction has been found to occur in hepatocellular carcinomas growing *in vitro*.

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PLATE

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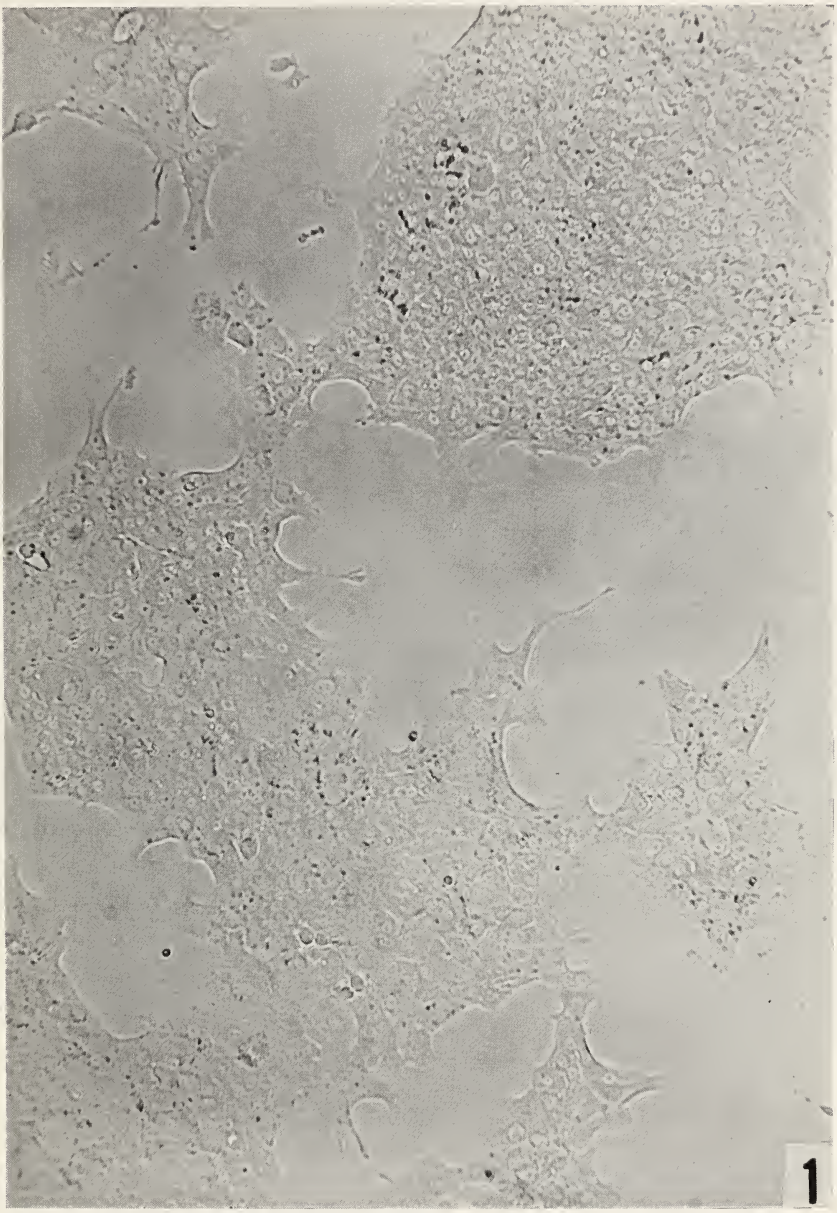
PLATE 20

FIGURE 1.—Photomicrograph of culture of H4-II-E. Approximately × 400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GLYCOGEN PHOSPHORYLASE IN HELA CELLS[1, 2, 3, 4]

JOSEPH B. ALPERS,[5] Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

Our interest in the glycogen phosphorylase of HeLa cells arose when we observed rather marked fluctuations in the endogenous glycolysis of these cells (*1*). Glycogen was identified as the carbohydrate store utilized by the cells, and the variations in endogenous glycolysis corresponded to variations in glycogen. These in turn were traced to the particular growth conditions used. If supplementation of the medium at the 5th day was such that at the time of harvesting 48 hours later glucose still remained in excess of 0.7 mM, substantial glycogen was present. With the final medium glucose at 0.2 mM or less, glycogen was exceedingly low. For convenience, cells grown so as to contain high glycogen were called "high-glucose" cells; the others, "low-glucose."

Corresponding to the increase in glycogen, increases in the enzymes directly concerned with glycogen in cell extracts were noted (*1*, *2*). The specific activity of phosphorylase in high-glucose cells was 3 to 10 times higher than the value in low-glucose cells. UDPG-glycogen transglucosylase (glycogen synthetase) activity was up to twofold higher. Moreover, phosphorylase in extracts of low-glucose cells was detectable essentially only in the presence of adenylic acid, whereas the enzyme in high-glucose cells exhibited a partial or variable dependence on this cofactor. None of the other enzymes of glycolysis, including phosphoglucomutase, was affected.

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[1] Presented at the Symposium on Metabolic Control Mechanisms in Animal Cells, Boston, Mass., May 27–30, 1963.

[2] Initial portions of these studies were performed at the Department of Biochemistry of the Public Health Research Institute of the City of New York, during which time the author was a postdoctoral fellow of the U.S. Public Health Service.

[3] This study was supported by grant A-5877 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service.

[4] *Abbreviations used:* AMP = adenosine monophosphate; ATP = adenosine triphosphate; IMP = inosine monophosphate; Pi = inorganic phosphorus; UDP = uridine-5′-diphosphate; UDPG = uridine-5′-diphosphate glucose.

[5] Recipient of a Research Career Development Award from the National Institute of Arthritis and Metabolic Diseases.

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MATERIALS AND METHODS

The HeLa cell line was obtained from Dr. B. Mandel, of the Public Health Research Institute of New York City, Inc. Cells were grown attached to glass in pint bottles and subcultured weekly. Two million cells multiplied to 20 million in 8 days. The medium (25 ml per bottle) consisted of Hanks' salts, with 0.1 percent yeast extract (Difco yeastolate) and amounts of serum as specified later. The initial glucose concentration was 5.5 mM. Penicillin, streptomycin, and mycostatin were included as previously described (*3*). The most striking increases in response to glucose occurred when cultures were grown in a medium containing 10 percent human serum and 20 percent horse serum, but the work reported here was with cultures grown without human serum but containing 20 percent horse serum and 0.1 percent lactalbumin hydrolysate. With this medium, low-glucose cells were produced when no supplementation was given. High-glucose cells were obtained by restoring the glucose in the medium to 5 mM 48 hours before harvesting.

Cells were harvested with Versene and washed as previously described (*3*). As before, to obtain extracts for enzyme assays, the cells were ground at 0° with glass beads in glycylglycine buffer, and the supernatant solution after centrifugation at 600 × *g* was used (*3*). Procedures for all analyses, including the spectrophotometric assay of phosphorylase in the direction of glycogenolysis and the measurement of glycogen synthetase by the incorporation of label from UDP-glucose-C^{14} into glycogen, have been described (*1*, *3*). Enzyme units are defined as μmoles of substrate converted per minute. Phosphorylase *b* kinase from rabbit muscle, a gift of Dr. E. Fischer, was freed of traces of phosphorylase *b* by precipitation at *p*H 5.7. All other reagents and enzymes were prepared or obtained as before (*1*, *3*). Details of the inactivation and activation procedures for phosphorylase have been given (*3*).

RESULTS AND DISCUSSION

Table 1 illustrates the increase in glycogen and its related enzymes when high-glucose cells were produced. The increase in phosphorylase was nearly fourfold; in glycogen synthetase, 50 percent. In nonsupplemented cells, successive daily measurements of glycogen from the 5th day confirmed the impression that glycogen was gradually declining from initially higher levels and that supplementation with glucose reversed this. If the growth medium was drawn off and replaced with a solution of buffered salts without glucose, the depletion of glycogen was accelerated and phosphorylase fell sharply in 12 hours. The specific activity of lactate dehydrogenase was unchanged. Conversely, a threefold increase of phosphorylase and a doubling of glycogen synthetase occurred within an hour following the exposure of low-glucose cells to a medium of buffered salts containing 10 mM glucose. Increases could in fact be observed within

TABLE 1.—Low-glucose and high-glucose HeLa cells

Cell preparation	Cell glycogen*	Specific activity†		
		Phosphorylase		Glycogen synthetase
		−AMP	+AMP	
Low-glucose	1. 4	0. 00	0. 21	0. 83
High-glucose	64. 0	0. 28	0. 79	1. 11

*μMoles glucose equivalents per 100 mg protein.
†Units per 100 mg protein.

15 minutes of exposure to glucose. Significantly, there was no added source of nitrogen in these experiments.

We were interested in other substrates that might promote these changes, and, using galactose, we found, as had Eagle and his co-workers, that this hexose is poorly utilized by HeLa cells (*4*). Table 2 shows that, when galactose was added to the glucose-depleted medium overlying 5-day cells, its consumption over the ensuing 48 hours was meager, although somewhat enhanced at the higher levels of galactose. In all cases, however, glycogen deposition was inordinately low. Table 3 gives results of an experiment in which equal amounts of glucose or galactose were added to the medium overlying 5-day cells. (The glucose that remained at this time was 0.3 mM.) The glucose supplementation was small, and was rapidly and totally consumed in 48 hours. Galactose, on the other hand, was poorly utilized, and most of it remained in the medium. But the cells grown in galactose exhibited essentially the same characteristics with respect to the glycogen and the enzymes as did the low-glucose cells. The experiment indicated that cells with low-glucose characteristics could be harvested from a medium containing 1.5 mM hexose. This behavior of HeLa cells with galactose became a useful means of producing cells with low-glucose characteristics, without subjecting them to conditions of actual hexose starvation.

In seeking an explanation of the observed increases of enzyme activity in response to glucose, we considered the *de novo* synthesis of enzyme protein unlikely. The response to glucose was rapid, *i.e.*, a matter of minutes to hours, but the cell generation time about a day. Also, an added nitrogen source was not required for the enzyme increases to appear.

TABLE 2.—Glycogen deposition in galactose-grown cells

	Galactose supplement* (mM)			
	2. 1	4. 0	5. 7	7. 4
Galactose disappearance†	1. 0	1. 2	1. 3	1. 7
Cell glycogen‡	0. 9	1. 3	0. 6	0. 5

*Introduced to the indicated concentrations 48 hours before harvesting.
† μMoles disappearing per ml medium.
‡ μMoles glucose equivalents per 100 mg protein.

TABLE 3.—HeLa cells grown in D-glucose or D-galactose*

	Glucose-supplemented	Galactose-supplemented
Final hexose (mM)	0. 0	1. 5
Hexose consumed	2. 4	0. 9
Cell glycogen†	1. 0	2. 8
Specific activity‡		
Phosphorylase (−AMP)	0. 06	0. 08
(+AMP)	0. 28	0. 28
Glycogen synthetase	0. 82	0. 99

*Forty-eight hours before harvesting, the medium (containing 0.3 mM glucose) was supplemented with additional glucose or galactose to 2.4 mM total hexose.

† μMoles glucose equivalents per 100 mg protein.

‡ μMoles per minute per 100 mg protein.

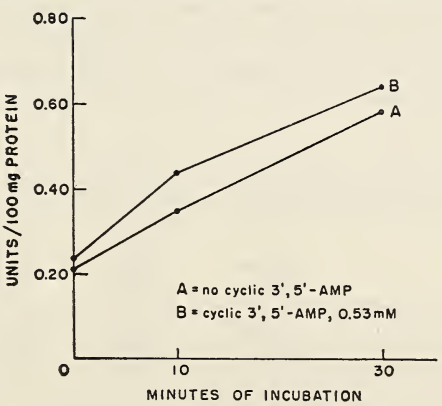
Ethionine and chloramphenicol did not interfere with the ability of the cells to respond to glucose, although such experiments, even if positive, would not conclusively rule out new protein synthesis.

The possible mediation by substrates of some protective role was investigated, since both the enzymes in question are known to be protected by one or more of the intermediates in glycogen synthesis. Glucose-1-phosphate added *in vitro* protects phosphorylase (*5*), and glucose-6-phosphate protects, as well as stimulates, glycogen synthetase (*6*). Moreover, both enzymes tend to occur in physical association with the "particulate glycogen" fraction of cells (*6*), which suggests that glycogen itself could be a factor in protecting the enzymes. The intracellular concentration of certain intermediates was therefore examined in perchlorate extracts of the two types of cells. Glucose-1-phosphate concentration was too low to detect by our methods, but glucose-6-phosphate concentration was about 0.1 mM in high-glucose cells and undetectable in low-glucose cells. UDPG in high-glucose cells was 0.12 mM, at least tenfold higher than in low-glucose cells. If the higher levels of intermediates in high-glucose cells were providing protection, this could be exerted either intracellularly or during the preparation of extracts. Experiments were accordingly performed in which low-glucose extracts were prepared in the presence of added excess substrates (glycogen, glucose-1-phosphate), but these did not alter the low-glucose characteristics. The possibility remained, however, that protection against proteolytic degradation might be exerted within high-glucose cells by the higher levels of intermediates that were observed.

Phosphorylase in various mammalian cells, especially liver and muscle, is known to be subject to activation and inactivation, and it was considered likely that the response to glucose in HeLa cells was also related to such phenomena. Crude extracts of HeLa cells were capable of converting muscle phosphorylase *a* to the *b* form, *i.e.*, to render it AMP-dependent. This degradative capacity was considerably greater in extracts of low-glucose cells. Various experiments implicated phosphorylase phosphatase as the responsible enzyme. The degradation of muscle

phosphorylase by HeLa cell extracts could be inhibited by 0.1 M fluoride, and also by mononucleotides such as 5′-AMP and IMP. (The latter reagents were effective only when inorganic phosphate was also included, which presumably protected the mononucleotides themselves against attack by deaminases and phosphatases.) The mononucleotides and fluoride are effective inhibitors of phosphorylase phosphatase both in mammalian liver and skeletal muscle (*7*).

In turn, the activation of HeLa cell phosphorylase was achieved in crude extracts, by use of an ATP-generating system and fluoride to inhibit phosphatase action. Text-figure 1 shows a threefold stimulation of the enzyme in an extract of low-glucose cells. This newly created activity was essentially independent of 5′-AMP. The increase was attributed to the action of a phosphorylase kinase on an inactive form of phosphorylase present in the crude extract. Cyclic 3′,5′-AMP, which would be expected to accelerate the early phase of the activation (*8*), showed essentially no influence, but was probably itself rapidly cleaved by the specific diesterase that is in crude tissue extracts. Phosphorylase activity in crude extracts of high-glucose cells could also be increased, but this was most prominently observed when a purified preparation of phosphorylase *b* kinase from rabbit muscle was included. Table 4 illustrates an experiment, in which an extract of low-glucose cells was activated over threefold, and the corresponding extract of high-glucose cells slightly over twofold. In both instances, the product of the kinase reaction was essentially independent of 5′-AMP. Note that the *total* activity detectable following activation with the kinase reaction remained higher in the high-glucose cell extract. This was true with all such experiments, and indicated that the state of *activation* of the phosphorylase could explain part, but not all, of the difference in phosphorylase between the two types of cells. Clearly, the *total* phosphorylase (active plus activatable) was higher in the high-glucose



TEXT-FIGURE 1.—Activation of HeLa cell phosphorylase. A crude extract of low-glucose cells was incubated at *p*H 7.0 in the presence of $MgCl_2$ and an ATP-generating system (phosphocreatine and creatine phosphokinase) at 30° C as described (*3*). A second incubation contained cyclic 3′,5′-AMP. Samples were removed at the indicated times for assay with 5′-AMP.

TABLE 4.—Activation of phosphorylase with added phosphorylase *b* kinase

Cell preparation	Incubation (min)	Specific activity*	
		−AMP	+AMP
Low-glucose	0	0. 05	0. 33
	15	1. 01	1. 15
High-glucose	0	0. 30	0. 70
	15	1. 54	1. 71

*Units per 100 mg protein.

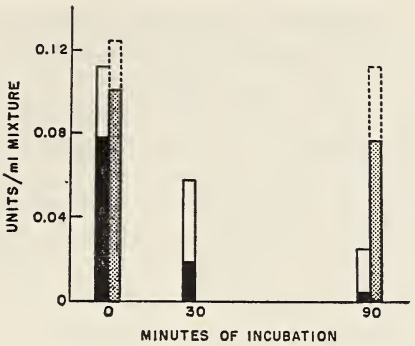
cells. Possibly the inactive enzyme in the low-glucose cells was more susceptible to further degradation, *i.e.*, proteolysis. In this connection, the increased phosphorylase phosphatase in low-glucose cells might serve to maintain more of the phosphorylase in an inactive, *i.e.*, labile, state.

Similar results have been obtained with partially purified preparations. Phosphorylase from HeLa cells has been partially purified, as shown in table 5, principally by a chromatographic technique with starch columns (*9*). The enzyme is specifically adsorbed by the amylopectin groupings of the starch, while inert protein comes directly through. After washing, the enzyme is specifically eluted with a dilute solution of glycogen. Text-figure 2 shows an experiment in which fully activated enzyme, thus purified, was degraded and reactivated. This preparation, activated and purified 125-fold, was exposed to a small sample of crude extract of low-glucose cells, which contributed negligibly to the measured (or potential) phosphorylase activity of the mixture. After 30 minutes' incubation, phosphatase activity in the crude extract had caused a 50 percent loss of total activity. After 90 minutes, only 20 percent of the activity remained, and virtually all of it was AMP-dependent. This material could, however, then be reactivated to a state nearly like the original by the addition of ATP, Mg^{++}, and phosphorylase kinase, plus fluoride to prevent phosphatase action. Such experiments with partly purified material leave little doubt that the phosphorylase of HeLa cells is subject to activation and inactivation, as is known for the enzyme in various other mammalian tissues.

TABLE 5.—Purification of a low-glucose extract*

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/100 mg protein)
Thawed extract	4. 0	0. 40	160	0. 25
After centrifugation	4. 0	0. 28	77	0. 36
After activation	7. 4	0. 78	77	1. 01
Column eluates				
#1	1. 0	0. 00		
#2	1. 0	0. 25	0. 72	31. 30
#3	1. 0	0. 00		
#4	1. 0	0. 00		

*Yield = 32 percent; purification = 126-fold.



TEXT-FIGURE 2.—Inactivation and reactivation of partially purified phosphorylase. Phosphorylase from low-glucose cells, activated and purified to a specific activity 126-fold above its original, was inactivated by incubating at 30° C with a crude extract of low-glucose cells. At 0 and 90 minutes, samples of the incubation mixture were taken for reactivation at *p*H 8.5 with an ATP-generating system and added phosphorylase kinase, as described (*3*). *Solid segments* indicate activity assayed without 5′-AMP; the total height represents the corresponding activity with 5′-AMP. Adjacent *stippled areas* at 0 and 90 minutes represent the activity of samples removed at these times and reactivated.

To understand the possible manner in which glucose in the medium might affect the level of active phosphorylase in cells, we must consider the mechanism of phosphorylase activation (*8*). It will be recalled that ATP is involved in several steps in the over-all process: *viz*, as a precursor of cyclic 3′,5′-AMP, and by directly donating a terminal phosphate for the activation first of phosphorylase kinase and then of the phosphorylase molecule itself. In this connection, the concentration of the various adenine nucleotides was examined in perchlorate extracts of cells. Table 6 indicates that high-glucose cells contained about twice the concentration of total adenine nucleotides in low-glucose cells and 7 times the concentration of ATP. No 5′-AMP was detected in the high-glucose cells, whereas this mononucleotide comprised about half the total adenine nucleotides in the low-glucose cells. These findings are quite compatible with the formulation that, under the growth conditions employed, ATP is the limiting factor for phosphorylase activation, and glucose activates the system by making ATP available. It is significant that the level of ATP observed in low-glucose cells is below the Km for ATP in the phosphorylase kinase reaction (4×10^{-4} M).

TABLE 6.—Cellular concentration of adenine nucleotides

Cell preparation	Glycogen*	Concentration (mM)			
		AMP	ADP	ATP	Total
Low-glucose	4	0. 48	0. 32	0. 24	1. 0
High-glucose	87	0. 00	0. 30	1. 59	1. 9

*μMoles glucose equivalents per 100 mg protein.

The importance of glucose in the present system may be understood in the following way: In low-glucose cells, metabolites significant for glycogen metabolism are in low concentration. This includes UDPG, glucose-6-phosphate, and ATP. Since phosphorylase and glycogen synthetase are both depressed, the over-all arrangement is suited for a state in which glycogen is neither being stored nor utilized to any extent. With the introduction of glucose, glucose-6-phosphate, UDPG, and ATP all increase. Glycogen deposition is stimulated by mass action from higher levels of the primary substrate UDPG; but probably most critical is the increase in glucose-6-phosphate, which is known to protect glycogen synthetase and especially to exert an allosteric stimulation by lowering the Km for UDPG (*10*). Studies recently reported by Hilz and coworkers (*11*), involving glycogen deposition in rat liver in response to cortisol, emphasized this strategic kinetic role of glucose-6-phosphate. Large, *i.e.*, 40-fold, increases of intracellular glucose-6-phosphate were revealed, which probably outweigh other influences in facilitating glycogen deposition, by this allosteric kinetic effect on the synthetase enzyme.

In high-glucose cells, the activation of phosphorylase, as we have stated, probably occurs as a result of the observed increase in ATP, which is involved in no fewer than three distinct sites in the process and is presumably limiting for the activation. But the net effect on glycogen deposition is not simple. Studies in other laboratories have shown that ATP can also alter the glycogen synthetase enzyme in various tissues in such a way that it becomes dependent on glucose-6-phosphate concentration (*10*, *12*). Were glucose-6-phosphate itself not simultaneously undergoing a marked increase, this action of ATP might result in a net depression of glycogen synthesis. When glucose is added, a new balance must become established involving the increased levels of the phosphorylase and synthetase enzymes, the substrates UDPG and Pi, and the special cofactors 5′-AMP and glucose-6-phosphate, with the net effect that glycogen is deposited. The correlations observed here suggest that in this tissue culture system, energy from glucose, *i.e.*, ATP, becomes the limiting factor governing at least one of the elements in this balance, *viz*, phosphorylase. Given the central role of ATP, this concept may bear implications for enzyme systems other than those involved with glycogen. The concept would also seem especially relevant to tissue culture cells, which, compared to living tissues, are subject to more arbitrary growth conditions.

SUMMARY

HeLa cells harvested from "high-glucose" medium had over 30 times higher glycogen than "low-glucose" cells and higher phosphorylase and glycogen synthetase activity. Other glycolytic enzymes were not affected. Cells grown on galactose resembled low-glucose cells. The increases in glycogen, phosphorylase, and glycogen synthetase were independent of added nitrogen in the medium. Ethionine and chloram-

phenicol did not interfere with the response to glucose. Low-glucose cells contained low levels of glucose-6-phosphate, UDPG, ATP, and total adenine nucleotides. Addition of glycogen or glucose-1-phosphate in the grinding medium did not affect phosphorylase activity. Extracts of low-glucose cells exhibited high phosphorylase phosphatase activity, which could be inhibited by fluoride or mononucleotides plus inorganic phosphate. Phosphorylase activity in crude extracts was increased by incubation with Mg^{++} and ATP, and was further stimulated by the addition of muscle phosphorylase *b* kinase. However, extracts of low-glucose cells did not reach the level of activity of high-glucose cells, when both were activated. Hence activation phenomena could account for much, but not all, of the observed differences. HeLa cell phosphorylase, activated and purified more than 100-fold, could be inactivated by crude HeLa cell extracts and then reactivated as described. It is suggested that ATP is limiting for phosphorylase activation in low-glucose cells. Added glucose activates phosphorylase by providing ATP.

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ENZYME CHANGES IN CULTURED HEART CELLS[1, 2]

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The study of differentiation and regulation in animal cells has involved the search for an adequate system. *In vivo* systems as represented by the developing embryo do not provide enough material or furnish sufficient experimental control. To overcome these difficulties, many investigators have turned to the use of tissue and cell cultures (*1*) and have had some success in demonstrating developmental changes in tissue culture (*2–5*). However, in almost all experiments the cell culture technique proved inadequate because single cells *in vitro* invariably lose their function after a given time. These losses by individual cells were termed "dedifferentiation" (*6*). Investigation of the process of dedifferentiation was carried out in various laboratories and in some instances the loss in function in the cell population either was due to the more rapid growth of unspecialized cells (*7*) or to a loss of function in the differentiated cells (*8–10*). In the latter case, the changes observed in differentiated cells grown in culture may reflect the effect of *in vitro* conditions on the delicate balance between metabolism and the functions characteristic of them. As such, these changing cells may yield a system whereby this balance, responsible for the specific function of the differentiated cell, may be investigated. Recently we have succeeded in culturing single beating heart cells from the postnatal rat heart (*11*). The beating in these cells may be maintained up to 6 weeks (*12*) after which it ceases, but the cells continue to grow. We have chosen to study this alteration of beating heart cells in culture because of the intrinsic advantage afforded by the beating phenomenon, which provides a measure of special function easily visualized, without manipulation, thus avoiding changes resulting from test situations. More specifically, in this system the process of so-called dedifferentiation or loss of specialized function may be attributed to changes in the heart cells themselves and not to changes in the nature of the cell population. We wished to determine some of the enzyme and metabolic changes, which accompany the loss of beating and specific heart proteins,

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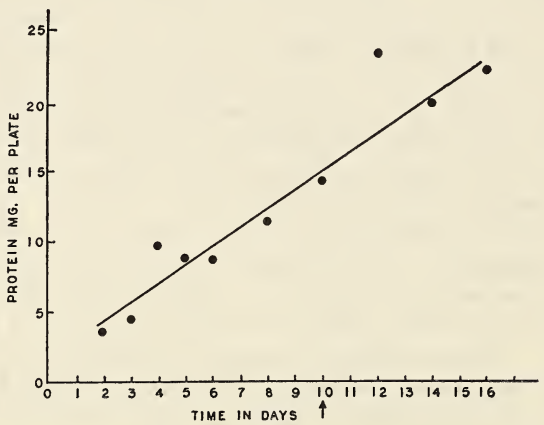
as a measure of specific function in cell cultures. Knowledge of such general and specific metabolic changes may then be used to determine the factors responsible for dedifferentiation in heart cell cultures and perhaps may lead to information of the factors controlling the specific function of a mammalian tissue.

GROWTH OF CELLS

The cells derived from young rat hearts were grown in a modified Puck's (*13*) and Eagle's (*14*) medium and observed for approximately 20 days. The conditions chosen are such that the cells cease beating at approximately 10 to 13 days.

Text-figure 1 contains a growth curve obtained by the determination of the total protein present on individual plates, harvested after the designated periods of growth as measured by the method of Lowry *et al.* (*15*). This curve shows that in the first 2 weeks of growth in culture the increase in protein was essentially linear. If the increase in protein is used as a measure of cellular division, it can be estimated that one division took place approximately every 4 days under these culture conditions. Independent cell counts of other cultures correlate with this observation. In many primary cultures, after 6 weeks the rate of cell growth indicates a doubling in 3 to 4 days.



TEXT-FIGURE 1.—Growth curve. Change in total protein, present in individual plates, with time.

CALCIUM ADENOSINETRIPHOSPHATASE (Ca-ATPase)

The specific activities of myosin and of enzymes representative of major energy-producing metabolic pathways were measured at various stages of growth *in vitro*. Table 1 lists the specific activities of Ca-ATPase, an enzymic activity of myosin, at various stages of growth. For

TABLE 1.—Changes in calcium adenosinetriphosphatase specific activity with time in culture*

Days in culture	μMole Pi per 5 minutes per mg protein
0	0. 97, 0. 87, 0. 74
2	0. 1, 0. 18, 0. 25
3	0. 38
4	0. 28
5	0. 55
6	0. 40
9	0. 35
11	0. 04
14	0. 01
15	0. 08
Line cells	0. 00

*Beating stopped on 10th day. We use the term "line cells" to describe cells that have been grown in primary culture for about 2 weeks and which are subsequentally trypsinized and transferred to other dishes. These transfers may be continued indefinitely. We have kept "line cells" growing for more than 2 years.

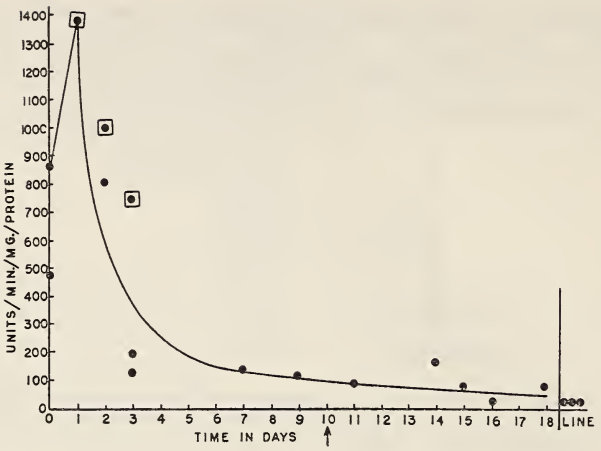
this determination, cells were washed with 0.154 M KCl, suspended in 0.50 M KCl and 0.2 percent ATP, homogenized and spun at 20,000 × *g* for 30 minutes. The activity was determined according to the procedure of Perry (*16*).

The Ca-ATPase activity decreased rapidly after trypsinization and plating. After the first few days when the cells stretched out and began to beat, the specific activity remained somewhat constant and then decreased rapidly after about 10 days to a very low level. The precipitous decrease occurred at the same time the beating stopped. It is unknown why there is a rapid decrease in Ca-ATPase specific activity after trypsinization and plating. The high ATPase activity in extracts of freshly trypsinized heart may be attributed to nonmyosin ATPase activity of contaminating cells, which do not adhere to the dish when cultured. However, independent of the initial drop, the activity remained high during the first few days and then decreased drastically when beating ceased.

Since the Ca-ATPase activity of line cells is quite low, possibly an inhibitor or inactivator of Ca-ATPase was present in these extracts. To test this possibility, extracts containing relatively high Ca-ATPase activity, prepared from intact hearts, were incubated with "line" cell extracts and the Ca-ATPase activity was measured. The result showed that there was essentially no effect of the line cell extracts on Ca-ATPase activity of the intact heart extracts. Apparently the low activities found in the line extracts and in extracts from nonbeating cells are not due to the presence of inhibitors or inactivators of Ca-ATPase activity.

ISOCITRIC DEHYDROGENASE AND MALIC DEHYDROGENASE

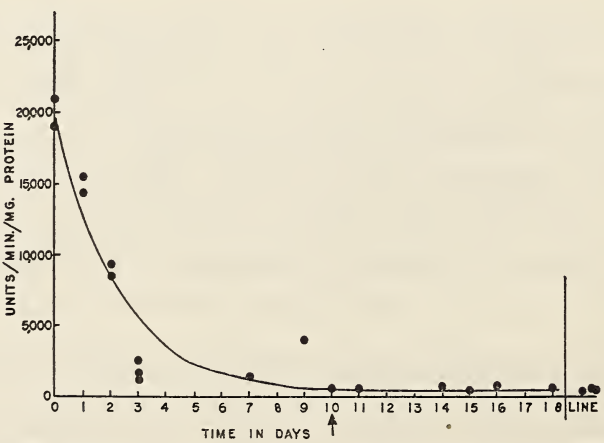
The specific activity changes of isocitric dehydrogenase and malic dehydrogenase are presented in text-figures 2 and 3. Extracts were



TEXT-FIGURE 2.—Change in isocitric dehydrogenase with time in culture. *Squares* and *solid circles* represent separate experiments.

prepared from cells homogenized in 0.15 M KCl and 1.6×10^{-4} M $KHCO_3$, *p*H 7.0, and centrifuged at 20,000 $\times$ *g* for 30 minutes. Activity was determined according to the procedure of Barban and Schulze (*17*). Initial experiments showed that extracts prepared either by centrifugation of the homogenates at 1000 $\times$ *g* for 10 minutes or at 20,000 $\times$ *g* for 30 minutes contained the same total activity for each enzyme. Therefore, the extracts obtained after centrifugation at 20,000 $\times$ *g* for 30 minutes were used for the determinations. Text-figures 2 and 3 show that there was a rapid decrease in the specific activities of both enzymes when cultured *in vitro*.

The presence of inhibitors or inactivators of either isocitric dehydrogenase or malic dehydrogenase in cultured cell extracts was tested by measurement of each dehydrogenase activity in the presence of cultured

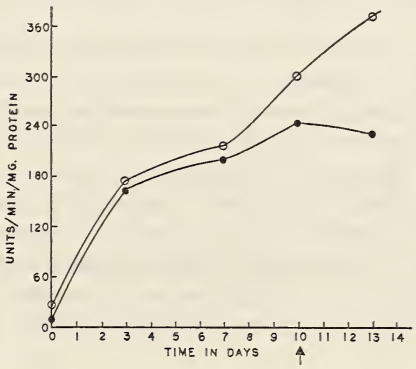


TEXT-FIGURE 3.—Change in malic dehydrogenase with time.

cell extracts incubated together with trypsinized cell extracts. Essentially no effect of the cultured cell extracts was observed on the activities of the trypsinized cell extracts. This indicates that the low isocitric dehydrogenase and malic dehydrogenase activities of cells grown in culture were not due to the presence of inhibitors of these two enzymes.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Text-figure 4 shows the pattern of glucose-6-phosphate dehydrogenase development in heart cell cultures. The extract was prepared in the same manner as for malic dehydrogenase, and the activity was determined by the method of Lieberman (*18*). The specific activity of this enzyme was low in trypsinized cells and after a few days in culture it increased to a level that was maintained through some 18 days in culture and in line cell extracts.



TEXT-FIGURE 4.—Change in glucose-6-phosphate dehydrogenase with time. *Open* and *filled circles* represent 2 different experiments.

Competition experiments with beating cell extracts in combination with intact heart extracts appear to rule out the presence of an inhibitor or inactivator of glucose-6-phosphate dehydrogenase in extracts possessing low glucose-6-phosphate dehydrogenase activities.

EFFECTS OF TRYPSIN

Since the changes in specific activities of the enzymes are based on comparisons with activities of freshly trypsinized cells, it was important to determine the effects of trypsin treatment on enzyme activities. Therefore, the enzymic determinations were carried out on baby rat hearts from which extracts were prepared before and after trypsinization. A comparison of the specific activities of the enzymes measured is shown in table 2, and the results indicate that trypsinization of the baby rat hearts did not change significantly the specific activities of the enzymes measured.

TABLE 2.—Effect of trypsin on specific activities of enzymes from baby rat hearts

Enzyme	Specific activity*	
	Before trypsinization	After trypsinization
Calcium adenosinetriphosphatase	0. 60	0. 86
Glucose-6-phosphate dehydrogenase	20	26
Isocitric dehydrogenase	810	873
Malic dehydrogenase	21, 900	19, 100

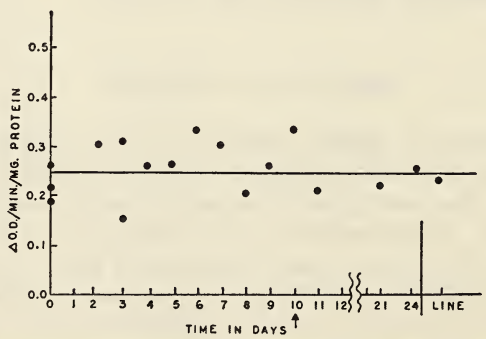
*See text-figures for definition of units.

HEXOKINASE

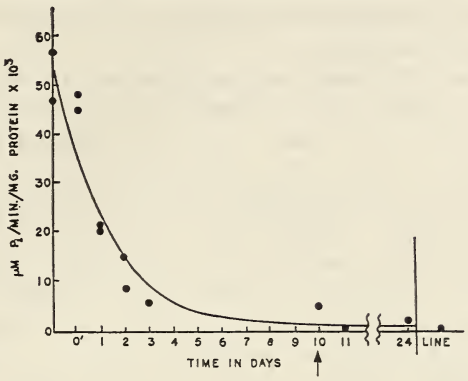
Initial experiments showed that extracts prepared either by centrifugation of the homogenates at 1000 × *g* for 20 minutes or 13,000 × *g* for 15 minutes contained the same specific activity at various stages of cell culture. Therefore, the extract obtained after centrifugation at 13,000 × *g* for 15 minutes was used for the determination (*19*), and the specific activity changes of hexokinase in the soluble fractions are presented in text-figure 5. The level of hexokinase remained constant through about 24 days in culture and in line cell extracts. Preliminary data, presently under further investigation, also suggest that the over-all rate of glycolysis remains fairly constant throughout the culture period. The specific activity of lactic dehydrogenase also appeared to decrease on culture, but to a much lesser extent than malic dehydrogenase, and it is surmised that since preliminary data indicate that lactate formation in culture does not change, lactic dehydrogenase is not limiting the rate of glycolysis.

CREATINE KINASE

As shown in text-figure 6, creatine kinase, an enzyme closely associated with the energy sources of contractile tissue, decreased in a similar manner to isocitric and malic dehydrogenase (*20*).



TEXT-FIGURE 5.—Specific activity of hexokinase during growth of heart cells in culture.



TEXT-FIGURE 6.—Change in creatine kinase activity with time in culture.

ENZYME CHANGES AND SPECIFIC FUNCTIONS

The demonstrations of the decrease in Ca-ATPase activity and disappearance of contraction of rat heart cells when grown in culture suggest that these cells lose their ability to contract because of a loss of actomyosin contractile function. The close relationship between Ca-ATPase and myosin (*21*), and hence of actomyosin, also makes this hypothesis reasonable. The decrease in Ca-ATPase activity is probably due to a loss of actomyosin function in the beating heart cells themselves and not to a rapid dilution of Ca-ATPase activity by nonbeating cells. Moreover, within the first 10 days, an examination of the plates reveals that the majority of the cells fixed to the plates are beating and are almost completely covering the plates. This also makes it unlikely that other types of cells could have grown out rapidly from such an environment. The evidence therefore indicates that a true dedifferentiation of heart cells may be taking place.

The decrease in specific activities of both malic and isocitric dehydrogenases indicates that the ability of the heart cells to oxidize substrates through the tricarboxylic acid cycle may be lowered. The decrease in the activities of mitochondrial enzyme activities was reported previously in other types of tissue culture (*22*).

Of all the enzymes examined in this present study, only glucose-6-phosphate dehydrogenase increased during passage of the heart cells in tissue culture and maintained its high activity over prolonged culture periods. Similar reports demonstrating the increase in glucose-6-phosphate dehydrogenase activity in tissue culture were made for other types of tissue (*23*, *24*). Since glucose-6-phosphate dehydrogenase is the first enzyme in the hexose monophosphate shunt pathway, the increase in the first enzyme may reflect an increase in shunt metabolism. The increase in glucose-6-phosphate dehydrogenase activity may reflect the need to maintain a relatively high intracellular reduced triphosphopyridine nucleotide (TPNH) concentration. This high TPNH concentration might be

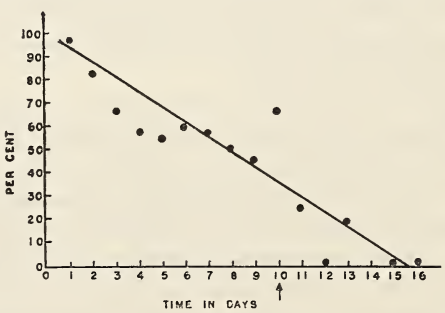
necessary for the synthesis of fatty acids or other intermediates utilized by the cells cultured *in vitro*.

Whether the line cells that eventually become established and can be maintained up to 2 years (*12*) are a result of a true dedifferentiation or of selective overgrowth is not clear. Our data indicate, however, that in the first few days of culture the specific function of the heart cells is maintained, as demonstrated by the maintenance of beating and the maintenance of Ca-ATPase. During this period, enzyme changes occur which may precede the loss of specific function. These preliminary changes may reflect a change in the control of metabolism to a type unable to maintain the morphological integrity of the cell. That the Ca-ATPase specific activity and beating are maintained during a period when other enzymes are changing, however, indicates that those changes are occurring in the heart cells and may very well be a part of the dedifferentiation process.

GLYCEROL-WATER EXTRACTED CELLS

Supportive evidence argues that functional changes occur in the heart cells. It is indicated from the study of single cells that an aging process occurs. Szent-Gyorgi contractile models (*25*) may be made by extracting glass-attached single beating cells in a glycerol-water solution (*26*). The cells retain their shape and visible structure as shown in figure 1a, but they no longer beat. These extracted cells may be contracted with ATP in the presence of Ca and Mg as shown in figures 1b, 1c, and 1d. The ability of beating heart cells to respond to ATP by contracting in this way diminishes with time. Dilute cultures were established so that they contained single beating cells not in contact with each other. The dilution was made low enough so that no growth or cellular division occurred. Under these conditions single cells may be seen that beat up to 6 weeks.

For 16 days beating cells were extracted and their reaction to ATP was tested. Text-figure 7 shows the percent positive contractions with



TEXT-FIGURE 7.—Percent positive contractions of extracted beating single cells. Each *point* represents the percent of positive contractions of about 80 separate attempts to cause contraction with ATP. At 16 days though each cell was beating before extraction, no cells contracted with ATP.

time, each point representing the percent positive contraction of up to 80 single beating cells. The loss of the ability to contract with ATP indicates a change of actomyosin properties with age and suggests a dedifferentiation process in the single cell, but this change does not immediately affect the beating.

LOCALIZATION OF CALCIUM ADENOSINETRIPHOSPHATASE

The process of dedifferentiation could be studied more adequately in the single cell if all the changes could be quantitatively observed on a microlevel. Thus, problems of variation in population and rate of division may be circumvented. Cognizant of this we are investigating the localization and estimation of enzyme activity in the single cell. Ca-ATPase may be measured in the single cell by a modification of the Gomori method (*27*). The adaptation consists of a selective extraction of the single cell and a substitution of ATP for glycerol phosphate in the Gomori technique.

Cultures of young rat heart cells attached to glass are extracted by the method of Hoffman-Berling (*26*), fixed at 0° C for 30 seconds with 3 percent calcium formol (*28*), and incubated for 15 minutes at 37° C in a mixture containing 10^{-3} M ATP, 6.7×10^{-3} M $CaCl_2$, and 2×10^{-2} M sodium barbital at *p*H 9.4. The calcium phosphate precipitate, resulting from the reaction of the inorganic phosphate formed at the site of ATPase action, is converted to a microscopically visible black deposit by conversion to Co S. Unextracted cells hydrolyze both glycerol phosphate and ATP. Extraction of the cells with glycerol-water solutions rendered them inactive with glycerol phosphate. As a control, inorganic phosphate was substituted for ATP in the reaction media. The result was an almost imperceptible over-all graying of the cells with no localization visible.

Figure 2 is a photomicrograph of a rat heart cell stained for Ca-ATPase activity; the blackened areas indicate the site of enzyme activity. The striations indicate that Ca-ATPase activity is discontinuously distributed along the myocardial fibril.

The Ca-ATPase activity of isolated myosin suggests that the activity in the myofibril may be associated with myosin. It would further suggest that the heavily stained band corresponds to the A band while the unstained area corresponds to the I band. It is unclear whether the alternating lighter stained bands are indicative of myosin at a region corresponding to the Z line or of some other calcium-activitated ATPase in the sarcomere. It is possible that the Ca-ATPase may be the result of the pumping of calcium by the sacrotubular vesicles, utilizing ATP. This activity would appear to be a calcium-activated splitting of ATP. During our study a similar observation was reported (*29*). The excellent electron micrographs demonstrate that Ca-ATPase is localized only in the A band. This may be further studied with the use of fluorescein myosin antibodies (*30*) in conjunction with enzymatic localization, and by selective inactivation of the calcium pump in the tubules.

Because of visualization of Ca-ATPase in the single cell, we can study the changes in the distribution of this enzyme during growth and dedifferentiation of single beating cells.

CAUSES OF DEDIFFERENTIATION

Probably the changes which occur are the result of an insufficient nutrition and an improper environment. The requirements for mammalian cells have been worked out in general terms for maintenance of many cell types established in culture, and are largely based on the ability to support growth. But the nutritional requirement for maintenance of function is still an unexplored area. Cells grown in a medium unable to support function may respond by changes in metabolism which serve to adjust to the new environment and permit survival, at the expense of the synthesis of specific proteins. The first step in this process may be a short period of metabolism of endogenous material during which time enzyme changes may occur.

We have some indication that this process is occurring in the heart cell. Cultures of heart cells will take up oxygen and continue to beat up to 36 hours without any added carbon source. The cells are undoubtedly metabolizing stored material and possibly utilizing structural and functional fats and proteins. In the presence of glucose in the medium, both endogenous metabolism and an adaptation to an altered metabolism may be occurring.

We have thus initiated a more general study of the metabolism of the heart cell in culture for information about over-all metabolic changes that may occur.

CHANGES IN O_2 UPTAKE AND RESPIRATORY QUOTIENT (RQ)

For the respiration studies, washed cells were suspended in isotonic buffer, *p*H 7.7, as used by Lehninger (*31*). After uniform suspension, the O_2 uptake and CO_2 production were measured in Dixon-Keilin vessels. Table 3 shows that O_2 uptake decreases with time in culture. An RQ of 0.81 at 0 days, which indicates mainly fat metabolism, changed gradually to an RQ of 0.96, indicating mainly carbohydrate metabolism, after 2 weeks in culture. In the beginning, addition of glucose shifted the RQ to 0.93 which may be the result of a shift of the metabolism from fat to carbohydrate. At 14 days, glucose addition shifted the RQ from 0.96 to 1.0, a much slighter shift.

Many investigators have presented evidence of the close relationship of fat oxidation to heart function. Fats are actively taken up by the myocardium (*32–35*), heart tissue actively metabolizes fat, and the normal heart RQ is around 0.75 (*36*). This indicates that the rat heart cells start from

TABLE 3.—Changes in RQ and O_2 uptake

Days in culture	O_2 Uptake (μl/hr/mg)			CO_2 Uptake (μl/hr/mg)		RQ	
	Endogenous	Endogenous plus glucose*	Change with glucose* (%)	Endogenous	Endogenous plus glucose*	Endogenous	Endogenous plus glucose*
0	18. 6	26. 1	+40	15. 1	24. 2	0. 81	0. 93
7	10. 3	8. 2	−20	9. 0	7. 8	0. 87	0. 95
14	6. 9	6. 3	−9	6. 6	6. 3	0. 96	1. 00

*Concentration of glucose added = 2×10^{-2} M.

a metabolic economy largely dependent on fat and shift, with time, in a glucose medium, toward one mainly utilizing carbohydrates.

Table 3 shows the effect of glucose on O_2 uptake changes during the shift. In the beginning, glucose is markedly stimulatory. After a week in culture, glucose becomes an inhibitor and a Crabtree effect is established. Table 4 gives 2 more examples of this. In the 2 weeks when the glucose effect changes, pyruvate remains an active stimulator of O_2 uptake. It has been maintained that the presence of a Crabtree effect is an indication of a control mechanism centered about glucose metabolism. This also applies to the Pasteur effect, the inhibition of glucose uptake by O_2 (*37–39*). Table 5 presents data indicating that the uncultured cells do not have a Pasteur effect but develop one with age in culture. It is tempting to speculate that, with the shift from fat to carbohydrate metabolism, controls concerned with carbohydrate utilization are brought into operation. Both the Crabtree and Pasteur effect seem to be established at the same time—about 3 to 4 days in culture.

EFFECT OF AMYTAL ON O_2 UPTAKE

Table 6 presents data on the effect of amytal on the O_2 uptake. Experiment A shows that a concentration of 10^{-3} M amytal inhibits O_2 uptake 49 percent. Although glucose stimulates O_2 uptake almost 50 percent, the inhibition by amytal in the presence of glucose is much greater, increasing as it does to 70 percent, and the value of O_2 uptake in the presence

TABLE 4.—Effect of glucose and pyruvate on oxygen uptake*

Days in culture	O_2 Uptake (μl/hr/mg dry wt)			Percent change	
	Endogenous	Endogenous plus glucose	Endogenous plus pyruvate	With glucose	With pyruvate
0	15. 3	17. 4	20. 8	+14	36
6	9. 7	8. 8	12. 0	−9	24
14	7. 4	6. 0	10. 0	−19	35
1	11. 8	14. 6	—	+24	—
6	9. 6	7. 8	—	−19	—
14	8. 5	6. 7	—	−21	—

*Concentration of additions: glucose = 2×10^{-2} M; pyruvate = 2×10^{-3} M.

TABLE 5.—Development of Pasteur effect

Days in culture	O_2 Uptake (μl/hr/mg)		Glucose uptake (μmoles/hr/mg)		Lactate formed (μmoles/hr/mg)	
	Endogenous	Endogenous plus glucose*	Air	N_2	Air	N_2
0	22. 0	22. 3	0. 93	0. 9	1. 05	1. 32
3	4. 0	3. 8	0. 48	0. 58	0. 89	0. 94
6	5. 2	4. 6	0. 50	0. 64	0. 85	0. 91
10	5. 3	4. 1	0. 65	0. 85	0. 70	1. 72
17	8. 8	5. 9	0. 44	0. 78	1. 00	1. 46
0	14. 6	18. 4	0. 28	0. 20	0. 62	0. 46
3	10. 8	11. 5	0. 44	0. 67	0. 84	1. 52
8	12. 7	9. 8	0. 58	0. 94	1. 04	0. 84
13	10. 0	7. 9	0. 58	0. 79	1. 11	1. 58
17	9. 7	7. 3	0. 47	0. 78	0. 81	1. 49

*Concentration of glucose added = 10^{-2} M.

of glucose and amytal is less than with amytal itself. In the presence of palmitate an even greater stimulation of O_2 uptake occurs. This stimulation, however, does not change the effect of amytal which causes the same inhibition as in the endogenous experiment. One interpretation of these results is that glucose shifts the metabolism through a flavine that is more sensitive to amytal inhibition. If this were true, then the effect of palmitate in stimulating O_2 uptake, but not changing the amytal effect, would suggest that the endogenous metabolism is going through the same flavine as involved in the oxidation of palmitate. This of course would indicate that fats are the endogenous substrates, in support of the RQ measurement. In 18 days the O_2 uptake falls, glucose inhibits O_2 uptake, and the ability of palmitate to stimulate O_2 uptake is lost. Furthermore, the inhibition by amytal is greater and is not affected by either glucose or palmitate. The absolute value of endogenous inhibition by amytal is close to that given by glucose plus amytal at 0 days. One may reason that, since the metabolism has shifted toward carbohydrate in the 18-day-old cell, amytal is inhibiting the more sensitive flavine associated with glucose metabolism. Thus, the addition of glucose would no longer affect an increase in sensitivity.

The loss of the ability of palmitate to stimulate O_2 uptake also supports the hypothesis that cells in culture have shifted from fat to carbohydrate metabolism. Together with the loss of palmitate stimulation is the loss of the ability of palmitate to shift metabolism to a system less sensitive to amytal. These results are also shown in experiments B and C of table 6. These experiments show that the loss in the ability of palmitate to stimulate O_2 uptake occurs at about the same time as the Crabtree effect.

In summary, we presented evidence that changes in the source of energy may be associated with changes in specific function in dedifferentiating heart cells. We intend to continue our investigation of the source of energy and the control of metabolism that is tailored specifically for the cell's function, as measured by the synthesis of specialized proteins.

TABLE 6.—Effect of amytal O_2 uptake

Experiment	Days in culture	Additions*	O_2 Uptake		Percent inhibition
			−Amytal	+Amytal	
A	0	None	17. 0	8. 6	49
		Glucose	24. 9	7. 6	70
		Palmitate	28. 4	15. 6	45
	18	None	8. 2	1. 6	80
		Glucose	6. 0	1. 1	81
		Palmitate	8. 2	1. 5	82
B	0	None	23. 0	11. 7	49
		Glucose	25. 3	8. 1	68
		Palmitate	35. 0	15. 0	57
	8	None	17. 6	5. 6	68
		Glucose	12. 6	3. 3	74
		Palmitate	17. 6	5. 5	69
	15	None	12. 0	3. 5	71
		Glucose	9. 0	3. 0	67
		Palmitate	11. 2	3. 1	72
C	0	None	22. 0	12. 3	44
		Glucose	24. 6	10. 1	59
		Palmitate	30. 5	16. 5	46
	8	None	13. 5	4. 2	69
		Glucose	10. 9	2. 8	74
		Palmitate	12. 8	3. 7	71
	15	None	9. 0	2. 2	75
		Glucose	6. 5	1. 6	75
		Palmitate	9. 0	2. 3	75

*Concentration of additions: glucose $= 2 \times 10^{-2}$ M; palmitate $= 10^{-3}$ M; amytal $= 10^{-3}$ M.

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PLATES

PLATE 21

FIGURE 1.—Contraction of a glycerol-extracted beating heart cell with 2×10^{-5} M ATP. The process takes from 1 to 5 minutes. × 185

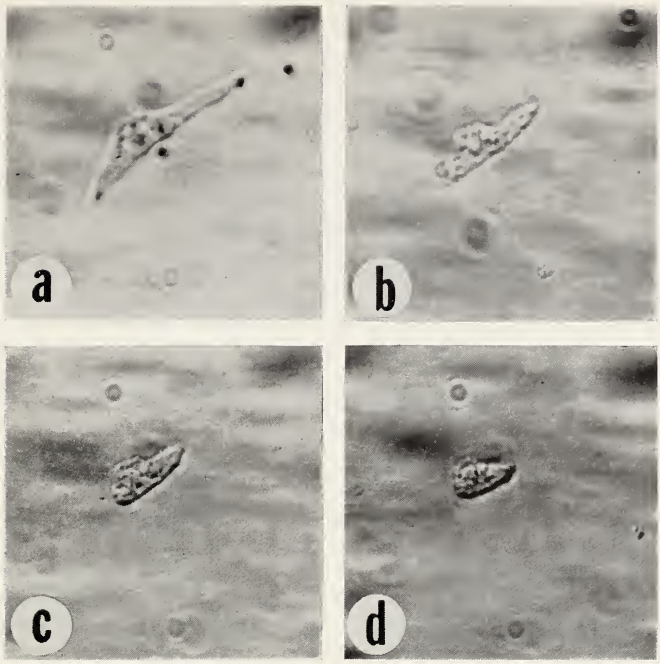
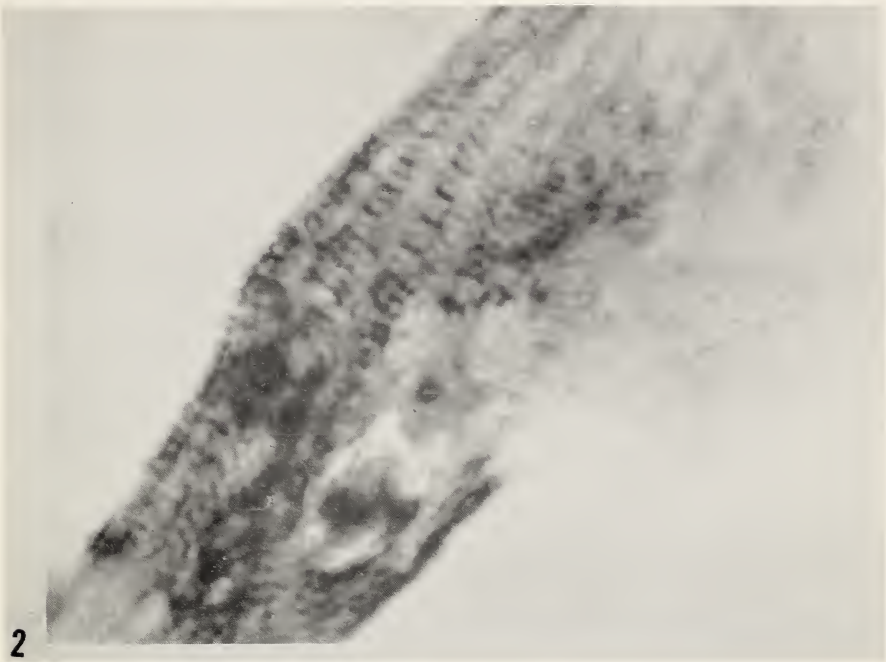

a
b
c
d

PLATE 22

FIGURE 2.—Bright-field photomicrograph of an extracted beating heart cell stained for calcium adenosinetriphosphatase activity. × 4,000



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